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The Role of Lck in Peripheral T cell Responses

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2/5/07

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Abstract

The signalling mechanisms that control differentiation of naïve T cells to effector and memory cells are unclear. A key event in T cell activation by antigen is the phosphorylation of tyrosine based activation motifs in the TCR CD3 complex by the Src-family kinases, Lck and Fyn. Elucidating the exact signalling mechanisms involved in the generation of memory T cells and effector function is fundamental and has important implications particularly in therapeutics, such as in developing treatments for diseases and infection.

Lck knockout mice have no T cells, and so we were unable to study the role of Lck in the peripheral T cell compartment. Using mice that express an inducible Lck transgene in T cells, which were bred to the class I MHC restricted F5 TCR transgenic line, we have investigated the role of Lck-mediated TCR signaling in antigen-specific CD8 T cell responses.

Stimulation of lymphocytes *in vitro* showed that the response of F5 T cells to peptide is 10-100 fold less sensitive in the absence of Lck, suggesting that the threshold of triggering is raised, however once cells were activated they underwent a similar program of division. Using an *in vivo* model where F5 T cells are transferred with flu virus into Rag1^{-/-} recipients, we demonstrated that Lck is required for the activation and subsequent generation of functional effector F5 CD8 T cells. The ability to generate functional Cytotoxic T lymphocytes (CTL) was impaired, shown by the reduced killing of target cells *in*

vitro and *in vivo*. We have also shown defects in the production of IL-2, TNF α and Granzyme B, which appear to be Lck dependent. However there is a less stringent requirement for Lck in the production of IFN γ , showing varying levels of Lck requirement for eliciting effector function.

In summary we have shown that Lck contributes to multiple stages of memory cell formation. It is required for the priming, expansion and differentiation of F5 CD8 memory T cells, but is not required for their survival.

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Abbreviations

% Percentage

-/- Homozygous knockout

μl Micro litre

°C Degrees Celsius

Ag Antigen

AIF Apoptosis Inducing Factor

APAF-1 Apoptosis Protease Activating Factor – 1

APC Allophycocyanin

APCs Antigen Presenting Cells

B cell B Lymphocyte

Biotin Biotinylated

BMDC Bone Marrow Derived Dendritic Cell

C Constant Segment

Ca²⁺ Calcium

CD Clusters of Differentiation

CFSE Carboxy-Fluorescein Diacetate Succinimidyl Ester

CLIP Class II associated invariant Chain Peptide

CMV Cytomegalovirus

CO₂ Carbon Dioxide

Csk	Carboxy-terminal Src Kinase
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T Lymphocyte Antigen – 4
CTLL-2	Cytotoxic T cell Line –2
⁵¹Cr	Chromium - 51
D	Diversity Segment
DAG	Diacyl Glycerol
DC	Dendritic Cell
DD	Death Domain
DISC	Death Inducing Signalling Complex
DOX	Doxycycline
DN	Double Negative
DNA	Deoxyribonucleic Acid
DP	Double Positive
EOMES	Eomesodermin
ER	Endoplasmic Reticulum
ERK	Extra-Cellular Signal Related Kinase
FACS	Fluorescent Activated Cell Sorting
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
Flu	Influenza

GDP	Guanosine Disphosphate
GEF	Guannine Nucleotide Exchange Factor
GTP	Guanosine Trisphosphate
HA	Haemagglutination
Het	Heterozygous
HI	Heat Inactivated
HLA	Human Leukocyte Antigen
Hom	Homozygous
IFN	Inteferon
Ig	Immunoglobulin
II	Invariant Chain
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IP₃	Inositol-1,4,5-triphosphate
IS	Immunological Synapse
ITAM	Immunoregulatory Tyrosine Based Activation Motif
ITIM	Immunoregulatory Tyrosine Based Inhibitory Motif
I.v.	Intravenous
J	Joining Segment
JAK	Janus-Activated Kinase

JNK	c-Jun N-Terminal Kinase
kB	Kilobases
kDa	Kilo-Daltons
LAT	Linker for Activated T cells
Lck	Lymphocyte Restricted Kinase
Lck^{ind}	Lck Inducible
LCMV	Lymphocytic Choriomeningitis Virus
LFA	Leukocyte Functional Antigen
LN	Lymph Node
M	Molar
MAPK	Mitogen Activated Kinase
MHC	Major Histocompatibility Complex
MFI	Mean Fluorescence Intensity
MLN	Mesenteric Lymph Nodes
mM	Milli Molar
μM	Micro Molar
mRNA	Messenger Ribonucleic Acid
MTOC	Microtubule Organising Centre
NFAT	Nuclear Factor of Activated T cells
NFκB	Nuclear Factor kappa B

NK	Natural Killer
NKT	Natural Killer T
ND	Not Detectable
NP68	Nuclear Protein 68
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
PIP₂	Phosphatidyl Inositol-4,5 Bisphosphate
PKC	Protein Kinase C
PLC	Phospholipase C
pMHC	Peptide Loaded Major Histocompatibility Complex
PTK	Protein Tyrosine Kinase
PTP	Protein Tyrosine Phosphatase
rtTA	Reverse Tetracycline Transactivator
SH-	Src Homology Domain
SLP-76	SH-2 Domain Containing Leukocyte Protein of 76kDa
SMAC	Supramolecular Activation Cluster
SPF	Specific Pathogen Free
SOS	Son of Sevenless
SP	Single Positive

STAT	Signal Transducer and Activator of Transcription
-------------	--

TAP	Transporters Associated with Antigen Processing
------------	---

T cell	T Lymphocyte
---------------	--------------

TCR	T Lymphocyte Receptor
------------	-----------------------

T_H	T Helper Cell
----------------------	---------------

TLR	Toll Like Receptor
------------	--------------------

TNF	Tumour Necrosis Factor
------------	------------------------

TRADD	TNF-Receptor Associated Death Domain
--------------	--------------------------------------

TRAF	TNF-Receptor Associated Factors
-------------	---------------------------------

T_{Reg}	Regulatory T Lymphocyte
------------------------	-------------------------

Tdt	Terminal Deoxynucleotide Transferase
------------	--------------------------------------

V	Variable Segment
----------	------------------

Vβ/α	Variable Region of TCR α or β
--	--

WT	Wild Type
-----------	-----------

ZAP-70	Zeta Chain Associated Protein Kinase of 70 kDa
---------------	--

Chapter 1: Introduction

1.1 Immune System

Vertebrates are constantly threatened by infection with microorganisms and have evolved systems of immune defence to eliminate infectious pathogens in the body. The mammalian immune system is comprised of two overlapping branches: innate and adaptive immunity. The innate immune system is the first line in host defence; the cells which function here, mainly originate from the myeloid lineage and include dendritic cells (DCs), macrophages, neutrophils, eosinophils, basophils, mast cells, and the natural killer and natural killer T cells (NK and NKT) which come from the lymphoid lineage. The cells of the adaptive immune system originate from the lymphoid lineage. These include B lymphocytes (B cells), which develop in the bone marrow and upon activation differentiate into antibody secreting plasma cells and T lymphocytes (T cells), which mature in the thymus and upon activation differentiate into armed effector cells, and carry out cell-mediated responses. Together and independently each arm of the immune system functions to combat and protect the host against infection.

1.2 Receptors of the Immune System

A number of key cell surface molecules and their interactions are required to control and augment antigen responsiveness in T cells. I will describe briefly

some of the important cell surface receptors involved in signal transduction in T cells.

1.2.1 T cell Receptor (TCR)

One of the key molecules on the surface of the T cell involved in antigen recognition and subsequent signal transduction is the T cell receptor (TCR). It is composed of the α and β polypeptide chains and its associated signal transduction components, CD3 (δ chain, γ chain, 2 x ϵ and 2 x ζ chains) (Reviewed in (Pitcher and van Oers, 2003)). The TCR complex itself lacks any intrinsic kinase activity, however the cytosolic components of the associated CD3 complex contain a unique motif, immunoreceptor tyrosine based activation motifs (ITAMs). This motif consists of six conserved amino acid residues spaced over approximately a 26-amino acid sequence, (D/E)X₇(D/E)X₂YX₂LX₇YX₂L/I, where X is any amino acid (Pitcher and van Oers, 2003; Reth, 1989).

A minority of T cells bear an alternative, but structurally similar receptor made up of a different pair of polypeptides termed γ and δ chains. $\gamma\delta$ T cells are more wide spread within epithelial rich tissues, such as the skin, intestine and reproductive tracts. Here they can comprise up to 50% of the T cells and their development can be thymic dependent or independent (reviewed in (Carding and Egan, 2002))

Generation of TCR Diversity

The antigen-binding portion of the TCR is known as the variable region (V) (Hozumi and Tonegawa, 1976). The TCR α chain is made up of three gene segments, the amino terminal variable (V), joining (J) and the constant (C) regions and the TCR β chain is composed of V, diversity (D), J and C gene segments. For a functional TCR to be expressed, DNA rearrangements of these gene segments must take place by a process known as somatic DNA recombination. To mediate this each segment is flanked by conserved non-coding DNA sequences known as the recombination signal sequence (RSS) (Akira et al., 1987; Hesse et al., 1989). The complex of enzymes that act to carry out somatic V (D) J recombination is termed V (D) J recombinase (Yancopoulos et al., 1986). The products of two genes, recombination-activating genes (RAG-1 and RAG-2) comprise the lymphoid specific components of the recombinase and this pair of genes is essential for V (D) J recombination. Mice in which either of the RAG genes is knocked out suffer a complete block in lymphocyte development at the gene rearrangement stage (Mombaerts et al., 1992; Shinkai et al., 1992). For further genetic diversity the addition of nucleotides in a template independent manner to the N regions at the V-D and V-J junction is achieved by the Terminal-deoxynucleotide transferase (Tdt) (Cabaniols et al., 2001).

1.2.2 Co-receptors

T cells have been divided into two major classes, which are distinguished based on their expression of cell surface glycoproteins CD4 and CD8. The co-

receptors of the antigen specific TCR participate in the development and selection of immature thymocytes (Chang et al., 2000; Itano and Robey, 2000), and in mature T cells recognise invariant portion of the major histocompatibility complex (MHC) class I and II molecules (described below in section 1.2.3).

CD4 is a single polypeptide, folded into four, external immunoglobulin – like domains (D1, D2, D3 & D4). D1 and D2 are packed tightly together forming a rod like structure, which are joined to D3 and D4 via a flexible hinge region (Brady et al., 1993; Ryu et al., 1990; Wang et al., 1990). CD8 is a disulphide bonded heterodimer of two polypeptides α and β . Both the polypeptides have an immunoglobulin like amino terminal domain linked to the membrane by a segment of extended polypeptide chain (Zamoyska, 1998). This segment is heavily glycosylated to help maintain an extended conformation and protect it from cleavage by proteases. CD8 α chains can also form homodimers but these are not expressed on the surface when CD8 β chains are present (Zamoyska, 1998). The co-receptors and TCR can bind the same peptide MHC complex, and both CD4 and CD8 can associate and recruit the protein tyrosine kinase Lck which in turn recruits Zap70 (Kim et al., 2003; Thome et al., 1995; Thome et al., 1996). Therefore upon interaction of the TCR, co-receptors (CD4 or CD8) and their respective peptide MHC complex Lck is brought into close proximity of the TCR complex and the kinase activity of Lck can initiate an intracellular signalling cascade.

1.2.3 Major Histocompatibility Complex (MHC)

In cellular immune responses antigens, generally peptides, are displayed to $\alpha\beta$ T cells in complex with class I or class II MHC molecules, which are encoded in the host genome as a large cluster of genes. CD4 and CD8 T cells differ in the types of MHC molecules they recognise; CD8 T cells bind to Class I MHC molecules and CD4 molecules bind to Class II MHC molecules. Both classes of MHC are heterodimers, the Class I MHC molecule is composed of two polypeptide chains, a larger α chain and a smaller non-covalently linked β_2 microglobulin chain, which is not polymorphic and is not encoded in the MHC locus. The Class II MHC molecule consists of a non-covalent complex of two chains (α and β) which both span the membrane. Both chains of the MHC Class II molecules are encoded in the MHC locus. MHC I molecules are found on nearly all nucleated cells, whereas the MHC II molecules are found only on the cell surface of specialised antigen presenting cells (APCs) like DCs and B cells. Crystallographic studies show that both the MHC molecules are folded in the same way. The major differences lie in the peptide-binding groove. In MHC Class II molecules the binding cleft is more open than in class I molecules. In the MHC Class I molecules the ends of the peptide is more buried in the molecule than in Class II molecules. Peptides, which bind to the MHC Class I molecules, are usually 8-10 amino acids in length, those which bind to the MHC Class II molecules are at least 13 amino acids long but can be much longer (reviewed in (Rudolph et al., 2006)).

1.2.4 Co-stimulators

When the TCR in conjunction with CD4 or CD8, interacts with MHC/Peptide presented by a given APC, the T cell becomes activated. However in addition to TCR engagement by peptide, ligation of co-stimulatory molecules are also required for a productive immune response to occur.

CD28

CD28 is a 44kDa member of the immunoglobulin superfamily and is constitutively expressed on T cells as a di-sulphide linked homodimer (Martin et al., 1986). The first suggestion that CD28 plays an important role in co-stimulation came from *in vitro* experiments showing its engagement with ligands CD80 (B7.1) and CD86 (B7.2) expressed on the surface of professional APCs could enhance IL-2 production (Jenkins et al., 1991). Further evidence from knockout mice showed the importance of CD28/CD80/86 interactions in T cell responses. While CD28^{-/-} mice retained the ability to combat some viruses, most T cell responses were severely impaired (Shahinian et al., 1993) and in the CD80/86 knockout mice T cells responses were absent (Borriello et al., 1997). Early studies on CD28 which demonstrated that CD28 provided a potent signal for the transcription and translation of several cytokines, following T cell activation (Thompson et al., 1989; Weiss et al., 1986). Thus it has been clearly demonstrated that the interaction of CD28 with ligands CD80 and CD86 is absolutely required for naïve T cell responses.

CTLA-4

A homologue of CD28 was discovered named cytotoxic T lymphocyte antigen-4 (CTLA-4). It was found that CTLA-4 could also bind the same ligands CD80/86 as CD28 but with much higher affinity, by a factor of 50-2000 fold (Greene et al., 1996; Linsley et al., 1994). Unlike CD28, CTLA4 is not expressed in naïve T cells, but is induced upon activation (Lindsten et al., 1993). CTLA-4 binds CD80/86 and delivers an inhibitory signal to the activated T cell. This makes the activated T cells less sensitive to stimulation by the APC and limits the production of the autocrine growth factor IL-2. CTLA-4/CD80/86 engagement is therefore essential in limiting the proliferative response of activated T cells to antigen. This was demonstrated by analysis of CTLA-4 deficient mice, which developed a fatal lymphoproliferative disorder (Chambers et al., 1997). T cell activation was detected in these mice 5-6 days after birth, and the mice died by day 18-20 because of lymphocytic infiltration into the non-lymphoid tissues. However thymocyte development in these mice appears normal, with no evidence of a failure to negatively select thymocytes expressing TCRs with high affinity for self-pMHC, instead suggesting that CTLA-4 is therefore required in peripheral T cell tolerance and homeostasis.

CD40L

CD40 is a type I transmembrane protein, constitutively expressed on many cells, including B cells, DCs, monocytes and macrophages. CD40L by contrast is expressed predominantly on activated CD4 T cells but expression on mast cells, basophils, eosinophils, NK cells and activated B cells has also been

reported (Clarke, 2000). CD40 and CD40L are important regulators of a variety of humoral and cellular immune responses (Gray et al., 1994; Grewal et al., 1995) and have a critical role in T cell dependent B cell responses. In the absence of CD40-CD40L interactions B cell clonal expansion, germinal cell formation, isotype switching and the generation of B cell memory are all defective (Gray et al., 1994). Defects in CD40-CD40L interactions also have an effect on *in vivo* priming and clonal expansion of antigen specific CD4 T cells (Grewal et al., 1995; van Essen et al., 1995). Ligation of CD40 by CD40L is thought to regulate CD4 T cell priming by influencing APC costimulatory activity. CD40L is rapidly up-regulated on CD4 T cells after TCR/MHC engagement on the surface of an APC. *In vitro* data shows cell surface expression of CD40L occurs within 2-4 hours of TCR ligation or stimulation with anti-CD3 antibody, peaks at 6-8 hours and is gone between 24-96 hours (Roy et al., 1993). The interaction induces the expression of costimulatory molecules and cytokines on APCs that are required for the full activation and differentiation of CD4 T cells (Clarke, 2000). These molecules include CD80, CD86, CD58 (LFA-3), CD54 (ICAM-1), as well as upregulating class I and class II MHC expression (Clarke, 2000).

ITIM containing Receptors

Both B and T cells receive signals that counteract and modify the activating signals delivered through antigen receptors and co-receptors. These inhibitory signals usually block the response by raising the threshold at which signal transduction can occur. Most of these modifying signals are received through

receptors that bear a distinct motif called the immunoreceptor tyrosine based inhibitory motif (ITIM) in their cytoplasmic tails. In this motif a large hydrophobic residue such as isoleucine or valine occurs two residues upstream of a tyrosine followed by two amino acids and a leucine to give the amino acid sequence ...[I/VXYXX:]... (Ravetch and Lanier, 2000). Ligand engagement by inhibitory receptors results in ITIM phosphorylation by Src and recruitment of protein tyrosine phosphatases (PTPs), such as SHIP, SHP1 and SHP2. PTP recruitment can result in decreased phosphorylation of activation pathway effectors, such as PLC γ , SLP-76, LAT, VAV and others.

1.2.5 Cytokine Receptors

Cytokines are major mediators of the immune response and control many different cellular functions. They are synthesised following various stimuli by different cells of the innate immune response such as monocytes, macrophages, DCs and cells of the adaptive immune response, the B and T cells. Cytokines elicit their effects by acting through specific receptors and subsequently signal through Janus Kinase/signal transducer and activator of transcription (JAK/STAT) pathways. Here I have described the structure and function of a few of the common cytokine receptors.

Common Cytokine Receptor γ Chain Family

The common cytokine receptor γ chain family, are a group of receptors in which each receptor complex is composed of two or three subunits, with one of those subunits being the common cytokine receptor γ chain (CD132). The common γ

chain receptor is a critical component of receptors for IL-2, IL-4, IL-9, IL-15 and IL-21 (Reviewed in (Kovanen and Leonard, 2004)).

IL-2 Receptor

The common γ chain (γ_c) was first identified as the third component of the IL-2 receptor (Takeshita et al., 1992a). The IL-2 receptor is composed of three subunits, α (CD25), β (CD122) and γ_c . The β and γ chain are functional components of other cytokine receptors (Giri et al., 1994; Kawahara et al., 1994) and the α chain is unique. The 3 receptor chains are not constitutively expressed on mononuclear cells and the affinity of the receptor is dependent on which subunits are expressed on the surface of the cell (Hatakeyama et al., 1989; Ohashi et al., 1989; Robb et al., 1987). The β and γ_c chains together form an intermediate affinity receptor (Takeshita et al., 1992b), and converts to a high affinity receptor upon expression of the α chain which is induced in T cells upon TCR engagement (Arima et al., 1991). IL-2 binding induces correct expression of the tertiary receptor complex for IL-2 receptor activity (Syed et al., 1998). The IL-2R itself lacks any intrinsic enzymatic activity, however upon engagement activates protein kinases, which phosphorylate tyrosine residues in the β and γ_c chain (Asao et al., 1992; Benedict et al., 1987; Mills et al., 1990). One of the major outcomes of IL-2 receptor signal transduction is the activation of JAK/STAT signalling pathways. Upon activation these transcription factors traffic to the nucleus where they cause upregulation of target genes (Reviewed in (Ellery and Nicholls, 2002)).

IFN γ Receptor

The IFN γ receptor is composed of two ligand binding IFNGR1 chains associated with two signal transducing IFNGR2 chains. IFNGR1 and IFNGR2 belong to the class II cytokine receptor family and are single pass transmembrane proteins (Reviewed in (Langer et al., 2004)). The IFNGR2 chain is ubiquitously expressed and is generally the limiting factor in IFN γ responsiveness, as the IFNGR1 chain is usually abundantly expressed (Bernabei et al., 2001). Both the IFNGR chains lack any intrinsic kinase/phosphatase activity and so associate with signalling machinery in order to mediate signal transduction. The IFNGR1 intracellular domain contains binding motifs for the JAKs and STATs (Reviewed in (Schroder et al., 2004)). Phosphorylation of the IFNGR1 intracellular domain on Tyr⁴⁴⁰ leads to the recruitment of STAT1 (Farrar et al., 1992). The intracellular region of IFNGR2 contains binding motifs for the recruitment of JAK2 kinase, however it is not tyrosine phosphorylated during signal transduction (Kotenko et al., 1995). The JAK and STAT1 binding motifs are required for receptor phosphorylation, signal transduction and induction of biological responses (Schroder et al., 2004).

TNF Receptors

The TNF receptor (TNFR) family are type I transmembrane glycoproteins and are characterised by the presence of multiple cysteine rich repeats in the extracellular amino terminal domain (Hohmann et al., 1989). TNF α binds to two distinct TNFRs called TNFR1 and TNFR2, which are expressed on all cell types except red blood cells (Hohmann et al., 1989). TNFR1 is ubiquitously

expressed whereas TNFR2 is more abundant on endothelial cells and cells of the haematopoietic lineage (Brockhaus et al., 1990; Porteu et al., 1991). After ligand binding TNFRs mediate either cell apoptosis or proliferation, and TNFR1 has been shown to mediate apoptosis and TNFR2 has been shown to mediate proliferation (Mukhopadhyay et al., 2001; Weiss et al., 1998). The TNFR family have no enzymatic activity and so upon activation recruit one or more TNFR-associated factors (TRAFs) in order to transduce a signal. TRAF 2 in particular is known to bind TNFR1 through TNFR-associated death domain (TRADD) protein (Li et al., 2002). Together the recruitment of these adapter proteins can activate signalling pathways involving nuclear factor kappa B (NF κ B), JUN N-terminal kinase (JNK), p42/p44 mitogen activated protein kinase (MAPK) and p38 MAPK (Aggarwal, 2003).

1.3 Peripheral T cell Signalling

Many studies over the years have focused on how a T cell is activated, and what components are required to translate extracellular signals delivered through the TCR into the nucleus. It has been shown that Lck and Fyn, members of the Src family protein tyrosine kinases (PTK) are the first signalling molecules downstream of the TCR to become activated upon TCR engagement with the pMHC complex. It is the activation of these signalling proteins, which lead to the initiation of various signalling cascades and ultimately to the recruitment of transcription factors, resulting in the regulations of genes involved in various outcomes including differentiation, proliferation and other

effector functions of the T cell. I will describe in detail the role of Lck in T cell signalling, and briefly some of the pathways it influences.

1.3.1 Immunological Synapses

Upon recognition of a target cell by a T cell, surface membrane proteins are rapidly reorganised and those at the contact site form a specific topological rearrangement called the immunological synapse (IS). The antigen specific interaction with the APC leads to the rapid recruitment of the cytoskeletal proteins LFA1 and talin to the cell contact, which stabilises the cell interaction between the T cell and APC. The microtubule organising centre (MTOC) is also rapidly reoriented to this point. Similarly proteins involved in the signalling cascade such as CD4, CD28, TCR, Lck and PKC θ are also concentrated at the IS region (Kupfer and Singer, 1989; Kupfer et al., 1987). Three-dimensional studies showed that these proteins were clustered into spatially segregated domains called SMACs (Monks et al., 1998). The TCR and the protein kinases are located in the central c-SMAC and the cytoskeletal proteins are found in the peripheral p-SMAC. The observation that Lck and Fyn are found in the c-SMAC along with engaged TCR suggested that SMAC formation is important for T cell activation. Data supporting this hypothesis, has shown engagement of TCR with altered peptides failed to induce productive T cell activation and also the induction of SMACs (Monks et al., 1998). However recent data has questioned the role of SMACs in signalling where it was shown the TCR mediated tyrosine phosphorylation occurred primarily at the periphery of the synapse and was reduced by the time the IS had formed (Lee et al., 2003; Lee

et al., 2002). The data suggested that the IS provided a surface allowing the activation and engagement of receptors other than the TCR. For example cytokine signalling and signalling pathways which are up-regulated after TCR triggering such as CTLA-4 may be facilitated by synapse formation (Linsley et al., 1992).

1.3.2 Src Kinases

The Src family PTKs were first discovered by Peyton Rous; who identified Src as a viral gene product from the Rous Sarcoma Virus (RSV). It was also shown that Src had kinase activity and the ability to induce cell transformation (Bernstein et al., 1976). Furthermore studies showed that Src was a member of a family of structurally related kinases. To date 9 members of the Src family have been identified, including Lck and Fyn (Thomas and Brugge, 1997). Lck was first discovered in murine lymphoma cell lines (Casnellie et al., 1983; Marth et al., 1985; Voronova et al., 1984), and was shown to be an important PTK with particular abundance in the T lymphocyte compartment (Marth et al., 1987). Fyn formally known as Syn was discovered in human cDNA libraries as a result of its sequence similarity with Src (Kawakami et al., 1986; Semba et al., 1986).

Structure of Lck and Fyn

Lck and Fyn are 56 and 59kDa proteins, respectively, with a domain organisation related to other members of the Src family PTKs (**Figure 1.1**). Both molecules have, an N-terminal attachment domain (unique domain), a Src

homology 3 (SH3) and SH2 domain, a tyrosine kinase domain, and a C-terminal negative regulatory domain. The N-terminal sites are modifiable in order to allow the addition of fatty chains, including myristic and palmytic acid moieties. The myristoylation, palmitoylation and S-acetylation of the cysteine residues found in this site are required for targeting these proteins to the plasma membrane and lipid rafts which are important in T cell signalling (Kabouridis et al., 1997; Koegl et al., 1994; Shenoy-Scaria et al., 1993; Timson Gauen et al., 1996; Wolven et al., 1997). The unique domain of Lck has a specific modification; a dicysteine motif, which allows its association to the CD4 and CD8 co-receptors. This interaction, facilitates Lck's participation in signal transduction (Shaw et al., 1990; Turner et al., 1990). Lck and Fyn are targeted to different cellular compartments. Lck is localised at the plasma membrane whereas Fyn associates with intracellular structures such as the mitotic spindle (Ley et al., 1994). By virtue of their SH2 and SH3 domains they can act as adapter molecules and through the SH2 and SH3 domains Lck and Fyn can facilitate the recruitment of other molecules required in T cell signalling. The kinase domain is responsible for the phosphorylation of ITAM residues.

Src Kinase – T cell Activation

Early studies in a T lymphocyte somatic mutant cell line (J.CAM1) supported an important role for Lck in TCR signal transduction (Goldsmith and Weiss, 1987; Straus and Weiss, 1992). J.CAM1, cells are defective in the induction of tyrosine phosphorylation, and this was shown to be due to a defect in the expression of functional Lck because of a splicing defect in the protein (Straus

and Weiss, 1992). During TCR-pMHC interactions, Lck is recruited to the TCR complex through its non-covalent interactions with CD4 or CD8 co-receptors. Upon clustering of the co-receptors during antigen recognition by the TCR it is thought that the Lck molecules transphosphorylate the activation loop of Lck (Tyr 394). As a result of this clustering active Lck is brought into close proximity of the TCR/CD3 complex, and is able to phosphorylate the ITAM residues of the ζ homodimer and the CD3 complex. Leading to the subsequent activation of various signalling cascades culminating in the transcription of genes required for full T cell activation.

Lck adopts specific conformations dependent on its activation status. The crystal structure of active Lck was solved along with inactive Src and subsequently a mechanism of activation was proposed (Sicheri and Kuriyan, 1997; Xu et al., 1999). The structural studies suggested that when the C-terminal Y505 residue was phosphorylated Lck adopted a closed conformation. The pY505 residue created an intramolecular binding motif between the SH2 and the kinase domain, rendering the kinase region inaccessible. This model was suggested to be true for both Lck and Fyn (Sicheri and Kuriyan, 1997). In contrast to this, when the phosphorylation of the activation loop of the Y394 residue in the kinase domain of Lck occurs it renders the protein fully active. This priming of Lck enhances the active formation and further increases its catalytic activity. The interactions of the SH2 and SH3 domains as well as the phosphorylation status of the activation loops control the conformation and activity of these kinases. Other crystallographic studies showed that

transphosphorylation between Lck molecules were prohibited when the Y505 residue was phosphorylated {Tanaka, 1986 #502; Xu et al., 1999}. Furthermore deletion or mutation of the Y505 residue resulted in a constitutively active form of Lck (Marth et al., 1988).

1.3.3 Signalling Cascades

A key-initiating event in T cell activation by antigen is the phosphorylation of ITAMs in the TCR subunits by the Src family kinases Lck and Fyn (**Figure 1.2**) (Gauen et al., 1994; Straus and Weiss, 1992). Lck is constitutively associated with the cytoplasmic domains of CD4 and CD8 co-receptor molecules (Barber et al., 1989; Veillette et al., 1988) and optimal signalling through the TCR occurs by clustering of the TCR and its co-receptors. Aggregation of the TCR with the appropriate co-receptor helps to activate the cells by bringing Lck together with the ITAMs and other targets associated with the cytoplasmic domains of the TCR complex. One of the principle targets of Lck is another kinase called ZAP-70 (Gauen et al., 1994), whose role is essential in propagating the signal downstream (**Figure 1.2**). The most important consequence of ITAM tyrosine phosphorylation is to create docking sites for the tandem SH2 domains of ZAP-70 in a highly specific and co-operative fashion (Wange et al., 1993). ZAP-70 once bound to the TCR is activated by the phosphorylation of the kinase domain activation loop (Tyr-493) mediated by the Src family kinases (Chan et al., 1995). Following activation ZAP-70 phosphorylates adapter proteins such as the linker for activation of T cells (LAT), SH2 domain containing leukocyte phosphoprotein of 76kDa (SLP-76)

and PLC γ (Fukazawa et al., 1995; Wardenburg et al., 1996; Zhang et al., 1998a). Adapter proteins lack either enzymatic or transcriptional activities, but are capable of mediating non-covalent protein-protein interactions with other signalling molecules via the tyrosine based signalling motifs or modular protein-protein interaction domains (eg. SH2 and SH3). The major function of adapter proteins is to facilitate the formation of multicomponent signalling complexes that allow the initial signal to be transduced from the cell surface into the intracellular environment.

LAT

LAT is a type I transmembrane protein, and contains two cysteine residues, which upon palmitoylation localise it to the plasma membrane (Lin et al., 1999; Zhang et al., 1998b). The phosphorylation of cytoplasmic residues within the transmembrane adapter protein LAT allows the binding of other molecules via their SH2 domains including PLC γ , GADS and Grb2 (Finco et al., 1998; Lin et al., 1999). The association of LAT and GADS in turn leads to the recruitment of SLP-76 and associated proteins to the LAT complex at the plasma membrane (Finco et al., 1998; Lin et al., 1999).

PLC γ

PLC γ directly binds phosphorylated LAT, its binding is essential for T cell activation (Lin and Weiss, 2001; Yablonski et al., 1998). PLC γ is activated by tyrosine phosphorylation and in turn cleaves phosphatidyl inositol 4,5 bisphosphate (PIP $_2$) generating inositol 1,4,5 trisphosphate (IP $_3$) and diacyl

glycerol (DAG) (Nishibe et al., 1990). DAG activates protein kinase C (PKC), and the isoform of PKC which plays an important role in mature T cell signalling is PKC θ . PKC θ has been shown to be important in TCR induced activation of NF κ B (Sun et al., 2000).

IP₃, Ca²⁺, NFAT

IP₃ generated following TCR stimulation binds to receptors in the endoplasmic reticulum membrane, opening Ca²⁺ channels that release Ca²⁺ into the cytosol (Guse et al., 1993; Imboden and Stobo, 1985). The elevation of intracellular Ca²⁺ is essential for T cell activation. A downstream target following Ca²⁺ influx is the Ca²⁺/calmodulin-dependent phosphatase calcineurin, which dephosphorylates nuclear factor of activated T cells (NFAT) family members. NFAT proteins contain N-terminal regulatory domains that contain serine rich regions (Klemm et al., 1997), which in the resting state of the cell are phosphorylated. NFAT resides mainly in the cytoplasm, after cell stimulation calcineurin dephosphorylates these serine residues revealing a nuclear localisation signal (Klemm et al., 1997) and NFAT translocates to the nucleus. In addition a Ser phosphorylation site has also been uncovered which is essential for the activation of the transcription activity of NFAT (Garcia-Rodriguez and Rao, 2000; Okamura et al., 2000). NFAT1 and NFAT2 are essential for gene expression of a number of genes, such as IL-2, IL-4, CD40L and FasL (Peng et al., 2001).

Ras and ERK

DAG leads to the activation of RasGRP (Ebinu et al., 2000), and activated RasGRP directly interacts with Ras and induces guanosine diphosphate (GDP)/guanosine triphosphate (GTP) exchange. Ras can also be activated via a PLC γ independent pathway by a guanine nucleotide exchange factor (GEF), called SOS (Egan et al., 1993). Ras-GTP activates the ERK MAP kinase pathway via the activation of MAPKKK, Raf. Raf leads to the phosphorylation of MAPKK, MEK1 and MEK 2. MEK1/2 in turn phosphorylate serine and threonine residues of ERK 1 and ERK 2. Activated ERK1/2 then translocate to the cell nucleus and can subsequently activate transcription factors such as Elk1, member of the Ets family of transcription factors. Elk1 activates the expression of c-fos, which hetero-dimerises with Jun family members forming an AP-1 complex a transcriptional regulator that can enhance NFAT binding to the IL-2 gene (Reviewed in (Iwashima, 2003)).

c-Jun NH2 terminal (JNK) Kinases

JNK kinases have been shown to be important in T cell activation and cytokine production. The JNK kinases are activated by MKK4 and MKK7 which themselves are activated by Rho family GTPases Rac and Cdc42. JNK kinases in turn activate Jun family members by phosphorylating serine residues in the N-terminal region of the proteins. c-Jun forms part of the AP-1 transcription complex (Karin, 1995).

p38 MAP kinases

p38 MAP kinases are important in T cell activation in response to environmental stress and pro-inflammatory stimulus, such as LPS. p38 MAP kinases are activated by MKK3, MKK4 and MKK6, which in turn can activate transcription factors such as ATF2, Elk1 and Fos among others. A recent study has linked p38 activities to Lck, which has been shown to phosphorylate Tyr323 residue of p38 resulting in its activation (Salvador et al., 2005).

1.3.4 Signal Termination

Following T cell activation, and mediation of a successful immune response, the activation signals need to be dampened down, leading to the reversion of an activated T cell to a resting state. A number of protein tyrosine phosphatases have a role in dampening the immune response these include, SHP1, LYP (mouse ortholog is called PEP (Matthews et al., 1992)), PTP-PEST, PTP-HCSF, LMPTP and PTPH1 (reviewed in (Mustelin et al., 2005)). Two of the key phosphatases and their functions will be described here.

SHP1

The most well studied of these phosphatases is SHP1, which contains an SH2 domain. SHP1 plays an important role in NK and B cells by regulating the signalling of inhibitory surface receptors, which possess an ITIM motif in their intracellular tails. After ligand binding ITIMs are firstly phosphorylated by Lck or Fyn and then recruit the tandem SH2 domains of SHP1. This binding activates SHP1 100-fold and juxtaposes it to its substrates, which include Y394 Lck

(Chiang and Sefton, 2001) and ZAP-70 (Brockdorff et al., 1999). SHP1 therefore functions to downregulate signal transduction pathways. This was illustrated by analysis of the SHP1 mutant mice (motheaten) (Tsui et al., 1993), which are runty and have a motheaten fur due to infiltration of activated phagocytes. They also develop autoimmunity and abnormal inflammation and pathology of many organs. Thymocytes from these mice are hyper – responsive to TCR stimulation (Pani et al., 1996), suggesting that SHP1 plays a role as a negative regulator of signalling during T cell maturation in the thymus. At least part of this function is mediated through a direct dephosphorylation of ZAP-70, resulting in tempering of downstream signalling (Brockdorff et al., 1999). A recent study suggested that an ITIM containing receptor is required for this, and CD22 expressed on T cells could inhibit T cell activation through SHP1 (Sathish et al., 2004).

LYP/PEP

Like SHP1, LYP and the mouse orthologue PEP is also expressed in the haematopoietic cells, including T cells, where it resides in the cytosol close to the plasma membrane (Gjorloff-Wingren et al., 2000). LYP/PEP acts on the positive regulatory Y394 residue of Lck (Gjorloff-Wingren et al., 1999), Y417 of Fyn and ZAP-70 (Mustelin et al., 1998). The dephosphorylation of Lck and Fyn by PEP is regulated by its association with the SH3 domain of Csk (Cloutier and Veillette, 1996; Cloutier and Veillette, 1999). LYP-Csk interactions have been shown to be important for the regulation of immune functions in humans, where a single nucleotide polymorphism in LYP that changes amino acid 620 from

arginine to tryptophan in the Csk binding motif markedly reduces Csk binding to LYP (Bottini et al., 2004). This point mutation is associated with increased incidence of autoimmune diseases such as type I diabetes (Bottini et al., 2004), rheumatoid arthritis (Begovich et al., 2004) and systemic lupus erythematosus (SLE) (Kyogoku et al., 2004). A negative regulatory role for PEP in TCR signalling was identified by analysis of *pep*^{-/-} mice. Positive selection is enhanced in thymocyte development in these mice, resulting in the clonal expansion of effector and memory T cell pools (Hasegawa et al., 2004). Effector T cells generated *in vitro* from these mice displayed increased and sustained phosphorylation of Y394 Lck and Zap-70 in response to TCR stimulation and increased proliferative responses (Hasegawa et al., 2004).

Src Kinase – Regulation

Adapter proteins are also shown to be important for the regulation of Src kinase activity. In the case of Lck and Fyn, a negative regulatory role has been proposed for the lipid raft associated transmembrane protein PAG/Cbp (Brdicka et al., 2000; Kawabuchi et al., 2000). Studies in cell lines and non-transformed human peripheral blood show that, PAG recruits the major negative regulator of Src kinases Lck and Fyn, the protein tyrosine kinase Csk to the plasma membrane (Brdicka et al., 2000; Kawabuchi et al., 2000). PAG recruits Csk to the plasma membrane and brings it into close proximity of Lck and Fyn in order to regulate their activity. Initial studies showed that PAG was constitutively phosphorylated and bound to Csk (Brdicka et al., 2000; Kawabuchi et al., 2000). Upon T cell activation, PAG is dephosphorylated and releases Csk.

The model from these studies suggest that the activity of Lck is kept low because Csk phosphorylates Lck (Y505), which is bound to phosphorylated PAG. After TCR/MHC-peptide engagement, PAG is dephosphorylated, thus releasing Csk. Following this, Y505 of Lck is dephosphorylated by CD45, protein tyrosine phosphatase. CD45 is highly expressed in T cells and dephosphorylation of Y505 of Lck permits the activation of the Src kinases and facilitates the initiation of TCR mediated signal transduction (Hermiston et al., 2003). The importance of CD45 activity in the regulation of Lck and Fyn was demonstrated with studies in CD45 deficient cell lines, which showed that Lck and Fyn were hyper-phosphorylated at their C-terminal negative regulatory tyrosine residues (Koretzky et al., 1991; Ostergaard et al., 1989; Stone et al., 1997). Similarly analysis of the CD45 deficient mouse showed few peripheral T cells due to TCR signalling in the thymus and therefore selection of SP thymocytes being severely affected (Byth et al., 1996; Kishihara et al., 1993).

1.4 Cytokine Signalling

The biological response of a cell to cytokines involves a complex network of signal transduction molecules. Signalling is initiated by the oligomerisation of cognate cytokine receptors expressed on the surface of target cells. The most well known intracellular signalling pathway that is activated by cytokines are two family of molecules the JAK proteins, which are cytoplasmic tyrosine kinases that constitutively associate with the cytokine receptors and the STAT transcription factors (**Figure 1.3**). Cytokines can also elucidate activation of the Ras-MAPK signalling cascade as well as the PI3 kinase pathway.

JAK/STAT

The JAK family are composed of four members Jak1, Jak2, Jak3 and Tyk2. Among the STAT transcription factors seven family members have been identified so far and are all located in the cytoplasm as latent transcription factors. STATs are recruited via their SH2 domains to phosphotyrosine motifs of activated receptors and subsequently become tyrosine phosphorylated by the Janus kinases. Phosphorylated STAT proteins dimerise and translocate to the nucleus where they act as transcriptional activators of specific genes.

One such example of the activation of the JAK/STAT signalling pathway is upon IL-2 receptor engagement. Despite lacking intrinsic catalytic activity IL-2R engagement activates protein kinases (Benedict et al., 1987) which phosphorylate tyrosine residues in the β and γ_c subunits (Asao et al., 1992; Mills et al., 1990) in a pattern distinct from that induced by TCR engagement (Saltzman et al., 1990). Lck shows increased activity following IL-2 stimulation in T cells and can mediate phosphorylation of tyrosine residues within the β subunit (Hatakeyama et al., 1991). The JAK kinases also interact with the IL-2R. In T cells Jak3 associated with the γ_c becomes activated and phosphorylated in response to IL-2 stimulation (Johnston et al., 1994; Witthuhn et al., 1994). Jak1 associates with the β subunit and is also activated in response to IL-2 (Miyazaki et al., 1994; Russell et al., 1994). It has been suggested that Jak1 and Jak3 together mediate the phosphorylation of tyrosine residues within the receptor subunits. Phosphorylated tyrosine residues enable

secondary signalling molecules to dock via their SH2 domains; these are activated by phosphorylation and couple receptor activation to downstream signalling pathways. For example this leads to the activation of the latent cytoplasmic transcription factors the STATs. STAT1, STAT3 and STAT5 are reported to be activated by IL-2 (Frank et al., 1995; Johnston et al., 1995; Nielsen et al., 1994). Studies show that Jak1 was able to phosphorylate STAT1, STAT3 and STAT5, but Jak3 could only phosphorylate STAT3 and STAT5 (Quelle et al., 1995; Witthuhn et al., 1999). STAT5 is involved in the induction of genes required for the cell proliferation and survival (Fujii et al., 1998; Lord et al., 2000; Yu et al., 1999). STAT3 in contrast is involved in upregulating genes, which are involved in driving cellular proliferation in response to IL-2, and it has also been described as an oncogene (Bromberg et al., 1999).

1.5 T cell Development

T cell development starts firstly in the fetal liver during embryogenesis and continues within the newly developed thymus that forms around embryonic days (E) 10-13.5 (Manley and Blackburn, 2003). Specific signalling molecules and transcription factors influence commitment to the T cell lineage. Notch-1, particularly is required for the development of the T cell lineage and was demonstrated by a study involving the over expression of a constitutively active *notch-1* by retroviral transduction (Pui et al., 1999). This led to a block in B cell development and the subsequent development of DP thymocytes in the bone marrow (Pui et al., 1999). In a converse study *notch-1* was conditionally

ablated in mice following birth and an early block in T cell development was shown (Radtke et al., 1999). The data demonstrated that Notch-1 pathway inhibits factors, which are responsible for B cell lymphopoiesis, whilst promoting development of the T cell lineage (MacDonald et al., 2001). GATA-3, transcription factor has also been shown to be important for the differentiation of the T cell lineage. It is widely expressed in all cells, however in haematopoietic cells it is restricted to the T cell lineage (George et al., 1994).

After commitment to the T cell lineage thymocytes develop through stages identified by the expression of cell surface markers and rearrangement of the TCR loci (**Figure 1.4**). Thymocyte development proceeds through four main stages, cells at each stage are distinguished based on their expression of the TCR CD4 and CD8 co-receptors. In the first stage of development thymocytes are double negative (DN) for both CD4 and CD8. At this stage of development thymocytes rearrange and express the TCR β chain.

Within the DN population T cell development can be further dissected via their expression of CD25 (IL-2R α) and CD44 (phagocyte glycoprotein, Pgp1) (Godfrey et al., 1993; Pearse et al., 1989). The DN1 population (CD25-CD44+) develops from common lymphoid progenitors entering the thymus from the bone marrow. Development proceeds to the DN2 stage (CD25+CD44+) with the upregulation of CD25 and the initiation of the rearrangement at the TCR β , γ , δ loci. DN3 cells (CD25+ CD44-) undergo selection for a successfully rearranged TCR β gene. Cells after this stage lead to the downregulation of

CD25 and the cells become DN4 cells. DN4 cells then differentiate into DP cells, and at this stage initiate the rearrangement of the TCR α locus, leading to the expression of a $\alpha\beta$ TCR.

Role of Src Kinases in T cell Development

Much of the early studies concerning Lck and signal transduction were conducted in cell lines, however more recently the functional role of Lck in thymocyte development and T cell activation was demonstrated by the analysis of the adult Lck^{-/-} mouse, which showed a profound block in thymocyte development at the stage of TCR β selection between the DN3 and DN4 stages, resulting in a reduction in thymus size and output of mature T cells into the periphery (Anderson et al., 1993; Molina et al., 1992). Only 5-10% of normal numbers of mature T cells are detected in the periphery of these mice (Molina et al., 1992). Lck-deficient thymocytes showed impaired allelic exclusion of TCR β chains, elevated expression of TCRs and a failure to express the CD5 molecule a marker of negative regulation in TCR mediated signal transduction (Tarakhovsky et al., 1995). Some thymocytes are able to progress through this developmental block to the double positive stage and were shown to be mediated by another member of the Src family of PTKs, Fyn (Groves et al., 1996; van Oers et al., 1996). Fyn-deficient mice however, show no block in pre-TCR signalling as the absolute numbers and frequencies of the different subsets in the thymus appear to be normal (Appleby et al., 1992; Stein et al., 1992). Yet a strict requirement for Src family kinases in thymocyte development was discovered when the Lck-deficient and Fyn-deficient mice were

intercrossed and the double knock mice showed a complete block in development at the DN3-DN4 transition stage (Groves et al., 1996; van Oers et al., 1996).

Following successful expression of the $\alpha\beta$ TCR, the DP thymocytes undergo a further checkpoint in which the TCR is selected based on its recognition of self-peptide in context of MHC (Fink and Bevan, 1978; Zinkernagel and Doherty, 1974). The purpose of this is to ascertain if the newly formed TCR will be able to recognise and bind foreign pMHC complexes. Thymocytes, which are unable to bind the self-pMHC complex die via neglect. Those, which weakly bind the self peptide – MHC complex, undergo positive selection and are rescued from cell death (Backstrom et al., 1998; Kisielow et al., 1988b). Thymocytes then finally differentiate into either CD8 SP or CD4 SP T cells dependent on their ability to bind MHC Class I and II molecules respectively. Finally those TCRs, which recognise and bind the self – peptide MHC complexes strongly undergo negative selection and die via apoptosis (Kappler et al., 1987; Kisielow et al., 1988a). Thus preventing potentially autoreactive mature T cells to migrate into the periphery. Fewer than 5% of thymocytes pass through these stringent checkpoints in selection (Cheng and Chan, 1997). Similarly $\gamma\delta$ T cells undergo VDJ rearrangement, but they differ from the conventional $\alpha\beta$ T cells because they are not MHC restricted, are able to recognise pathogens directly and reside mainly in the gut and skin (Carding and Egan, 2002). It has been shown that IL-7 is required for the development of $\gamma\delta$ T cells in addition to the $\alpha\beta$ T cells (Moore et al., 1996).

1.6 Peripheral T cell Activation

The first time a naïve T cell encounters a specific antigen is in the form of a pMHC complex on the surface of an activated APC. There are three main types of APCs, macrophages, B cells and DCs. For naïve T cells the most important antigen presenting cells are the highly specialised DCs, whose function is to ingest and present antigen. Upon successful recognition of antigen the naïve T cell becomes activated and is generated into an armed effector cell. Below is a synopsis of the mechanisms of antigen presentation by DCs to naïve T cells.

1.6.1 Dendritic Cells

DCs are professional APCs, which perform at the interface of the innate and adaptive immune systems. Immature DCs are produced in the bone marrow. FLT3 Ligand and GM-CSF are important growth and differentiation factors for DCs (Pulendran et al., 2001). When a DC interacts with microbial or viral products such as LPS, CpG DNA or RNA recognised by TLRs, the cell is triggered to undergo a process of maturation. This leads to the upregulation of costimulatory molecules CD80 (B7.1) and CD86 (B7.2) expressed on the surface of APCs, particularly DCs, and provide signals to naïve T cells for activation. DCs also produce important cytokines such as IL-12 and type I and II interferons and function to activate NK and NKT cells, which rapidly kill selective targets.

1.6.2 Mechanisms of Antigen Presentation

Infectious agents replicate in either of two distinct intracellular compartments. Viruses and certain bacteria replicate in the cytosol. Many pathogenic bacteria and parasites replicate in the endosomes and the lysosomes that form part of the vesicular system. Protein antigens are degraded into peptides inside cells are bound to MHC molecules and are subsequently delivered to the cell surface. Peptides from the cytosol are bound to MHC Class I molecules, whereas peptides generated in intracellular vesicles are bound to MHC Class II molecules. Here is a description of the mechanisms of antigen presentation by APCs.

MHC Class I Presentation

MHC Class I presentation of peptide is mediated by a family of protein transporters called transporters associated with antigen processing-1 and 2 (TAP1 and TAP2). These transporters mediate ATP-dependent transport of ions, sugars and peptides across membranes in many cells. Other constituents of the complex associated with TAP are tapasin, which stabilises the TAP1/2 heterodimer (Garbi et al., 2003), the soluble ER chaperone calreticulin (Wearsch et al., 2004), and the soluble thiol oxidoreductase Erp57 (Dick et al., 2002). Peptides are transported into the ER from the cytosol via TAP, and if required are trimmed by an ER-associated aminopeptidase (ERAAP and ERAP-1) to 8-10 amino acids in length (Saric et al., 2002; Serwold et al., 2002; York et al., 2002). If the peptide has the appropriate sequence, it binds to the MHC class I- β_2m heterodimer. The fully assembled MHC class I molecule

leaves the ER and travels via the Golgi apparatus to the plasma membrane, where it is accessible to CD8 T cells (Cresswell et al., 2005). In contrast to the classical mechanism of MHC class I antigen presentation in which cells present peptides from antigens the cell is synthesizing itself, APCs can also acquire and present externally derived antigens. Here the APCs internalize antigens from the extracellular environment and present them as MHC class I bound peptides. This process is termed cross presentation, and has been shown to be efficient at priming CTLs (Reviewed in (Shen and Rock, 2006)).

MHC Class II Presentation

MHC Class II molecule is assembled in the endoplasmic reticulum with the chaperon molecule invariant chain (Ii) (Cresswell, 1996; Sant and Miller, 1994). The cytoplasmic tail of the Ii contains a motif that targets the Ii-MHC Class II complex to the endosomal pathway. Following entry into the endosomal/lysosomal compartments, Ii is degraded by the lysosomal proteases, leaving a small fragment of Ii called class II-associated invariant chain peptide (CLIP) associated with the MHC class II peptide binding groove (Maric et al., 1994; Riese et al., 1996). Antigens, which are delivered to the endosomal/lysosomal network, are also broken down by proteases, yielding peptide ligands for class II molecules. The catalytic removal of CLIP and capture of antigenic peptides by MHC class II proteins is mediated by the MHC-encoded molecule human leukocyte antigen HLA-DM, the resulting peptide complex is trafficked to the cell surface (Denzin and Cresswell, 1995; Sherman et al., 1995).

1.7 T cell Subsets and Effector Functions

1.7.1 CD4 T cells

Following activation, naïve CD4 T cells differentiate into functional subsets; two of the most well described are called T helper type 1 (T_H1) and T_H2 cells. This nomenclature is based on their production of signature cytokines $IFN\gamma$ and IL-4 respectively (O'Garra, 1998). T_H1 cells are essential for the protection against various intracellular infections, including bacteria, parasites, yeast and viruses. T_H1 cells via their production of $IFN\gamma$ and lymphotoxin can activate microbicidal activity as well as cytokine production by macrophages. T_H1 response helps drive B cell differentiation and leads to the production of complement fixing antibodies of the IgG2a isotype, as well as the activation of NK cells and CD8 T cells expressing $IFN\gamma$ and perforin. T_H1 cells can also mediate immunopathology and have been implicated in autoimmune diseases such as type 1 diabetes and multiple sclerosis. T_H2 cells produce cytokines IL-4, IL-5 and IL-13 which can activate mast cells and eosinophils and can be protective against extracellular infections such as helminths and certain parasites. T_H2 derived cytokines can also mediate allergic responses and induce airway hyperreactivity as well as the production of IgE (Reviewed in (O'Garra and Arai, 2000)).

Recently additional T_H subsets have been identified which have effector functions distinct from the T_H1 and T_H2 subsets described. These include T_H3

cells, which produce TGF β , Follicular helper cells (T_HFH), peripherally induced T regulatory cells (T_{Reg}) and IL-17A producing (T_H17) cells. T_HFH were first identified in humans, and represent T cells found in germinal centers they selectively express CD84, CD200, Bcl-6 and IL-2 (Chtanova et al., 2004). Also a subset has been shown to express CXCR5 and migrate to B cell follicles after activation. T cell-B cell interaction at the follicular border is crucial for efficient B cell activation and for antibody production. Studies have suggested that T_HFH may be important in regulating autoantibody production (Vinuesa et al., 2005).

The T_H17 lineage has been linked to models of autoimmune diseases including EAE and collagen induced arthritis (Langrish et al., 2005; Murphy et al., 2003). This subset of T cells produce IL-17A, IL-17F, IL-6 and TNF α , and their function reflects the ability to mobilize, recruit and activate neutrophils. Recent studies suggest that T_H17 lineage arise separately and do not represent a population of T_H1 cells that have undergone further differentiation (Park et al., 2005; Veldhoen et al., 2006).

Regulatory T cells

Regulatory T (T_{Reg}) cells are a subset of CD4⁺ T cells, and play a crucial role in immunological tolerance, transplantation and autoimmunity (Sakaguchi et al., 1995). There have been many different types of T_{Reg} cells identified recently, of which the naturally arising CD4⁺ CD25⁺ T_{Reg} cells are the best studied and characterised. These cells comprise ~ 5-10% of the CD4⁺ T cells in the peripheral T cell compartment and represent a unique T cell lineage which

undergoes thymic selection and migrate into the periphery (Itoh et al., 1999; Papiernik et al., 1998).

The importance of T_{Reg} cells in immune regulation was first demonstrated by the depletion of this subset from CD4 T cells in a normal mouse, which resulted in the development of a number of autoimmune diseases upon transfer of purified CD4+CD25⁻ T cells into immuno-incompetent mice (Sakaguchi et al., 1995). The suppressive capacity of T_{Reg} cells has also been demonstrated in an *in vitro* assay in which naïve CD4 T cells were co-cultured with purified T_{Reg} cells, which suppressed the proliferation by specifically inhibiting the production of IL-2 (Thornton and Shevach, 1998). The mechanism by which T_{Reg} cells mediate naïve T cell suppression is said to be primarily cell contact dependent.

CD25 was traditionally used to identify T_{Reg} subsets however it is not a specific marker for naturally occurring T_{Reg} cells, since it cannot distinguish T_{Reg} cells from activated effector T cells. Recently the discovery of the Foxp3 transcription factor was shown to be selectively expressed by CD4+CD25⁺ T_{Reg} cells and was required for their development (Fontenot et al., 2003; Hori et al., 2003).

Since the discovery of naturally occurring T_{Reg} cells, a number of other T_{Reg} subsets have been identified. These include a subset, which develop in the periphery, and are often Foxp3⁻ and have been termed induced T_{Reg} cells (Apostolou et al., 2002; Zhang et al., 2001). Studies have suggested that the

cytokine TGF β may induce CD4+foxp3+ cells in the periphery from precursor naïve CD4+ foxp3- cells (Chen et al., 2003). Studies in mice, showed that TGF β converted T_{Reg} cells mediate suppression through cell-cell contact *in vitro* when co-cultured with normal CD4 T cells. *In vivo* these TGF β converted T_{Reg} cells could also inhibit the expansion of antigen driven CD4 T cells (Reviewed in (Vieira et al., 2004)).

CD4 T cell Help

Conventional CD4 T cells are also shown to be important in helping the CD8 T cell response in efficient pathogen clearance upon infection. This includes clonal expansion, development of effector function and the generation of long-term memory. Some groups have found CD4 T cell help can enhance priming of CTL responses, particularly for weak antigens (Franco et al., 2000; Wang and Livingstone, 2003). During priming, CD4 T cells can provide needed cytokines and growth factors such as IL-2 to facilitate the expansion of CTL during an acute response (Wang and Livingstone, 2003). Other studies have shown that the presence of CD4 T cell help in CD8 T cell priming is believed to be essential for the formation of CD8 T cell memory responses upon antigen re-challenge but was not required to mediate pathogen clearance in the primary response (Bourgeois et al., 2002a; Shedlock and Shen, 2003; Sun and Bevan, 2003).

The mechanism by which CD4 T cells can mediate this help is known to be through cellular interactions such as CD40/CD40L. Data has shown that

activated CD4 T cells can activate APCs in a CD40 dependent fashion, and strongly supports the view that CD4 T cell help for CTL priming is provided through the APC (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). Yang et al showed that induction of adenovirus specific CTLs 7 days post infection was impaired in mice treated with anti-CD40L antibody (Yang et al., 1996). In contrast to this study, acute infection with lymphocyte choriomeningitis virus (LCMV) in CD40L deficient mice generated a strong primary antiviral CTL response (Borrow et al., 1996). However their ability to maintain long-term control of virus replication and establishment of memory CD8 T cells was compromised (Borrow et al., 1996).

1.7.2 CD8 T cells and Effector Functions

Naïve CD8 T cells first encounter antigen in lymphoid organs, where it is presented by DCs (Bevan, 1976). Presentation by immature DCs results in the induction of tolerance, whereas the presentation by activated mature DCs stimulates the generation of CTLs and memory CD8 T cells (Moser, 2003; Steinman and Nussenzweig, 2002). DC maturation due to engagement of TLRs by pathogen products or by CD40 dependent interaction with CD4 T cells leads to an increased surface expression of antigen and CD80 and CD86 costimulatory ligands. This seems to be the basic requirement for mature DCs to stimulate an effective response. However recent evidence suggests that an additional signal is required to augment a strong signal for clonal expansion, such as the production of IL-12 and type I interferons (Curtsinger et al., 2003; Curtsinger et al., 1999; Curtsinger et al., 2005; Mercado et al., 2000). The

degree to which each of these signals influences the development of naïve T cells into effector cells and eventual memory T cells can vary. A number of groups have investigated the initial requirements for antigen stimulation provided by APC. Stimulation for as little as 2.5 hours, results in programmed cell division for at least 8 cell divisions and *in vivo* can result in a population of long – lived memory CD8 T cells (Kaeche and Ahmed, 2001; van Stipdonk et al., 2001; Wong and Pamer, 2001). However a recent study using 2 photon microscopy, suggested DC-CD8 T cell interactions occurred in distinct phases (Mempel et al., 2004). The initial T cell contact with DCs, occurring within the first 1-8 hours is a short lived transient interaction, resulting in T cells activation. This is followed by more sustained interactions lasting up to several hours (8-24 hour) in which the IS formation occurs between the DC and T cell, and a critical period of programming takes place (Mempel et al., 2004).

Antigen recognition initiates T cell proliferation that can be tightly coupled to changes in gene expression and importantly the differentiation into effector CTL. CTL gain the ability to produce antiviral cytokines such as $\text{TNF}\alpha$, $\text{IFN}\gamma$ and cytotoxic molecules, such as perforin and granzymes and rapidly eliminate the infectious pathogen (Bachmann et al., 1999a; Murali-Krishna et al., 1998; Oehen and Brduscha-Riem, 1998; Opferman et al., 1999; Veiga-Fernandes et al., 2000). Following antigen removal, a second phase ensues in which 90-95% of the CTL die via apoptosis and the surviving effectors differentiate into memory CD8 T cells (Murali-Krishna et al., 1998) (**Figure 1.5**).

1.7.3 Production of Anti Viral Effector Cytokines

Effector CD8 T cells contribute to pathogen clearance by elaboration of effector functions such as perforin dependent cytotoxicity and production of antiviral cytokines. In addition to the antiviral effects mediated by CTLs other cells also produce various cytokines such as the type I interferons IFN α and IFN β which are produced by many cells upon viral infection, and have potent antiviral activities. It has been suggested that IFN α and IFN β have immune regulatory functions including the stimulation of IFN γ production by CD4 T cells (Nguyen et al., 2002), and effects on Ag-presenting dendritic cells (Le Bon et al., 2003). Another pro-inflammatory cytokine IL-12, is also produced by DCs and induces the production of IFN γ . IL-12 is known to augment CTL responses in a number of experimental systems (Gately et al., 1991; Trinchieri, 1998), where addition of IL-12 to cultures with APCs results in strong clonal expansion and development of cytotoxic activity by naïve T cells. IFN α and IFN β have also been shown to provide this additional signal upon activation of CD8 T cells to stimulate clonal expansion and differentiation (Curtis et al., 2005). Here I will describe some of the key cytokines produced by effector CD8 T cells.

IL-2

Once naïve CD8 T cells have been activated they produce IL-2, and undergo 7-8 rounds of cell division over 3 days and differentiate to become cytotoxic effector cells (Reviewed in (Mescher et al., 2006)). IL-2 is implicated in the generation of effector phenotypes from antigen-stimulated cells and has been shown to be especially important for the proliferation of primary activated T cells

(Dai et al., 2000). IL-2 delivers its signal through the heterotrimeric IL-2R (Taniguchi and Minami, 1993; Wagner et al., 1980). A study by Cheng et al examined the effects of enhancing IL-2R signalling in CD8 T cell responses. A transgenic mouse model was developed with CD8 T cells capable of augmenting autocrine IL-2R signalling after target cell recognition. Data showed that after antigen stimulation *in vivo*, the transgenic CD8 T cells demonstrated marked increase in cell proliferation, expansion and continued increase in cell expansion following repeat antigen exposure. Therefore the group concluded the presence of augmented IL-2R signalling sets the size of the initial response to antigen (Cheng et al., 2002). In addition to IL-2's role in the proliferation of primary activated T cells, it has been proposed that IL-2 is also required for the secondary expansion of memory CD8 T cells (Williams et al., 2006). Studies by Williams et al adopted a mixed chimera strategy in which T cells lacking the IL-2R α chain could be studied in a healthy mouse. The group demonstrated a role for IL-2 signals during the primary infection in programming the development of memory CD8 T cells.

TNF α

TNF α is an important proinflammatory cytokine, which is produced by a variety of cell types, including activated CD8 T cells. TNF α has strong antiviral activity but can be toxic and even lethal to the host if produced chronically or at high levels. It has a variety of effects on cells expressing either one or both of its receptors from activation to cell death. TNF α can mediate apoptosis of virally infected cells by binding to TNF receptors, which leads to the activation of

transcription factors NF κ B and AP-1, and to the induction of proinflammatory and immunomodulatory genes (Herbein and O'Brien, 2000). The mechanism by which TNF α mediates apoptosis of its target cell through its receptor is explained in section 1.7.4.

IFN γ

IFN γ is a type II interferon and is produced by effector CD8 T cells, as well as T_H1 CD4 T cells, macrophages, NK and NKT cells, and is an important anti-microbial effector cytokine. IFN γ is important in host cell response to intracellular pathogens and functions to up-regulate the class I presentation pathway to increase the diversity of peptides expressed on the cells surface in the context of Class I MHC. The up-regulation of Class I MHC is important for pathogen clearance as it increases the potential for cytotoxic T cell recognition of foreign peptides and thus promotes the induction of cell-mediated immunity. IFN γ stimulation induces the replacement of constitutive proteasome subunits with immunoproteasome subunits. In unstimulated cells, the proteasome enzymatic subunits are β 1, β 2 and β 5, encoded outside of the MHC locus. IFN γ induces the expression of new subunits, LMP2, MECL-1 and LMP7, which competitively replace β 1, β 2 and β 5 of the proteasome, respectively (Schroder et al., 2004). New species of the proteasome are formed consisting of LMP2:MECL-1:LMP7 (the immunoproteasome), LMP2:MECL-1: β 5 or β 1: β 2:LMP7, as well as low levels of the β 1: β 2: β 5 constitutive proteasome (Schroder et al., 2004). Inducible proteasome replacement is thought to be a mechanism by which IFN γ can increase the quantity, quality and repertoire of

the peptides for MHC Class I loading. The cleavage specificity of the immunoproteasome may allow production of peptides better able to bind MHC class I molecules thereby increasing the efficiency of the system. Peptide diversity is thought to increase as a result of differences in cleavage specificities between different species of immunoproteasome. As a whole this serves to increase the levels and diversity of epitopes presented for CD8 T cell recognition in the context of MHC Class I and thus increase immune surveillance (Dick et al., 1996; Groettrup et al., 1996). This mechanism may have evolved to ensure LMP2/LMP7/MECL-1-dependent epitopes are only produced at sites of inflammation and thus avoid autoimmunity without compromising appropriate T cell stimulation. The efficiency of peptide generation is further increased with the IFN γ induced PA28, comprised of α and β subunits and associates with the proteasome and alters proteolytic cleavage. It is thought to enhance antigen processing towards a more efficient generation of TAP and class I MHC compatible peptides to increase the overall efficiency of Class I MHC peptide delivery (Dick et al., 1996; Groettrup et al., 1996). The IFN γ inducible TAP transporter is vital in peptide transport from the cytosol to the ER lumen (Schroder et al., 2004). TAP transiently associates with class I MHC to aid in efficient peptide loading (Schroder et al., 2004). IFN γ also up-regulates the MHC Class I complex, chaperones such as tapasin and GP96, which are implicated in the efficient assembly of peptide: MHC class I complexes (Anderson et al., 1994; Suto and Srivastava, 1995).

1.7.4 Cytotoxicity

The efficient removal of infected cells is an important process for host defence against viral pathogens, since majority of the viral life cycle occurs inside the cell. Therefore death of the infected cell is the most efficient and sometimes the only way to eliminate viral infections. CTL mediate lysis of target cells via apoptosis and in general is orchestrated by a family of proteases called caspases (Nicholson and Thornberry, 1997). CTL can induce caspase-mediated killing via one of two mechanisms, the granule exocytosis (perforin/granzyme) pathway and the Fas/FasL pathway.

Perforin and Granzyme Mediated Cytotoxicity

When CD8 T cells receive specific signals to activate and proliferate via the TCR, transcriptional mechanisms are activated that lead to the production of cytotoxic granules and their constituent proteins (Russell and Ley, 2002). Within a day after T cell activation, granules begin to be synthesized along with perforin and granzymes and other granule components and reside in the cytoplasm of the cell until target cell identification. Upon identification of an infected target cell, perforin polymerises, enters into the target cell membrane, and forms a pore allowing the delivery of granzymes into the target cell. CTL from perforin deficient mice are unable to induce killing via granule exocytosis and these mice have increased susceptibility to viral infections and tumours (Kagi et al., 1994; Kojima et al., 1994; Smyth et al., 2000; van den Broek et al., 1996). Granzymes are serine proteases, which upon entry into the target cell can induce cell death. Of the granzymes, granzyme B is a 32kDa protein and is

the most well studied and the only one shown to mediate caspase dependent killing (Trapani and Smyth, 1993). Granzyme B enters the target cell by endocytosis by binding the mannose-6-phosphate receptor (Motyka et al., 2000) or by fluid uptake (Trapani et al., 2003). Once inside the target cell granzyme B can cleave various intracellular substrates initiating a killing cascade. Granzyme B cleaves Bid a pro-apoptotic factor which is a member of the Bcl-2 family (Alimonti et al., 2001) to generate a truncated protein, which translocates to the mitochondrial outer membrane where in conjunction with Bcl-2 family members Bax and Bak causes mitochondrial outer membrane permeabilisation. This in turn triggers the release of several pro-apoptotic proteins including cytochrome c, initiates the formation of an apoptosome complex that activates the initiator apoptotic protease activating factor (APAF-1) and caspase-9, which then proteolytically cleaves and activates caspase-3 (Alimonti et al., 2001). This cascade of events leads to apoptosis of the target cell. Granzyme B is also able to directly cleave caspase 3 and caspase 8 (Atkinson et al., 1998; Medema et al., 1997; Yang et al., 1998).

Fas/FasL Mediated Cytotoxicity

Another extrinsic mechanism of killing infected target cells is through Fas (CD95) and FasL (CD95L) pathway. The Fas/FasL pathway is a mechanism by which apoptosis can be induced through the expression of death receptors on infected target cells. Engagement of Fas on the target with CTL expressed FasL results in the recruitment of the initiator caspase, caspase 8, though the interaction with the adapter molecule Fas-associated death domain protein

(FADD) by means of death domains and death effector domains to form death inducing signalling complex (DISC) (Peter and Krammer, 2003). This results in the activation of caspase 8. Caspase 8 is subsequently able to activate other members of the caspase family such as caspase 3 directly (Scaffidi et al., 1998). By contrast it is able to also activate Bcl-2 family member Bid, leading to the subsequent activation of caspase 9 through the interaction of APAF-1 as previously described.

Other Pathways for Mediating Cyotoxicity

There are several other death receptors that induce apoptosis, including those that belong to the tumour necrosis factor receptor (TNFR) family (Gupta, 2002; Gupta and Gollapudi, 2006). These molecules have a cytoplasmic death domain (DD), which does not have enzymatic or signalling activity and therefore must recruit a set of adapter molecules to activate the apoptotic process. $\text{TNF}\alpha$ mediates its signalling via TNFR1 and TNFR2 (Gupta, 2002; Gupta and Gollapudi, 2006). TNFR1 mediates both apoptotic and survival signals, whereas TNFR2 lacks DD and predominately mediates survival signals. $\text{TNF}\alpha$ induced apoptotic signal is provided by the recruitment of Fas associated death domain (FADD) to the TNFR1 DD and TNFR-associated death domain (TRADD) complex. FADD in turn recruits pro-caspase 8 by protein-protein interactions via a common death effector domain to form a death inducing signalling complex (DISC). During DISC formation, pro-caspase 8 is autocatalytically cleaved, activated and released into the cytoplasm, where it activates effector caspases (Caspase 3, 6 and 7). These effector caspases

cleave a number of cytoplasmic and nuclear substrates to induce apoptosis (Gupta, 2002; Gupta and Gollapudi, 2006). Caspase 8, in some cell types cleave a member of the Bcl-2 family, Bid and truncated Bid (tBid) translocates to the mitochondria and block anti-apoptotic property of Bcl-2/Bcl-X_L and induces apoptosis.

Death signals from the mitochondrial pathway of apoptosis do not require binding to cell surface receptors (Green and Reed, 1998). These signals include oxidative stress and growth factor withdrawal. Death signals induce depolarisation of mitochondrial membrane and release of cytochrome c and apoptosis inducing factor (AIF). Cytochrome c in the presence of ATP or dATP binds to an adapter protein Apaf-1, which recruits pro-caspase-9, which then activates effector caspases 3, 6, 7 to induce apoptosis. AIF in contrast translocates to the nucleus and induces large internucleosomal DNA fragmentation in a caspase independent manner.

1.8 Generation of Memory CD8 T cells

Immunological memory is the ability of the immune system to recall and then re-enact its previous encounters with specific antigens with improved speed and efficiency. Memory B and T cells contribute to this memory immune response, and this forms the basis of protective immunity. The accelerated recall responses of memory T cells to re-infection results from qualitative and quantitative changes in antigen-specific T cells (Kaech et al., 2002b). Compared to naïve animals, precursor frequency of antigen specific T cells can

be 100-1000 fold higher in immune mice (Ahmed and Gray, 1996). Memory T cells also exhibit rapid and efficient generation of effector function upon secondary challenge of a given pathogen. This function of memory T cells is attributable to re-programming of gene expression profiles by epigenetic changes (DNA methylation, histone modifications or reorganisation of chromatin structure). Studies have shown that memory cells are generally better equipped to assimilate TCR stimulatory signals (Kersh et al., 2003) and to mediate effector function.

Recently it has been shown that memory cells over express several important genes that control the transition between G₁ and S phase of the cell cycle. Two groups have shown high expression of cyclin dependent kinase 6 (CDK6) in memory T cells compared to naïve (Latner et al., 2004; Veiga-Fernandes and Rocha, 2004). Memory T cells have been shown to be pre-charged with a number of factors that are necessary for G₁-S phase transition which suggests that a lower threshold of stimulation is required for entry into cell cycle, and hence allowing rapid proliferation into effector cells (Latner et al., 2004; Veiga-Fernandes and Rocha, 2004).

1.8.1 Memory Cell Markers

Over the past few years different subsets of memory T cells have been identified. Parallel studies conducted in humans and mice identified these subsets based on their expression of cell surface markers. These include the expression of molecules required in cell adhesion, trafficking and chemokine

receptors such as CD44, the L-selectin CD62L and CCR7. CCR7 and CD62L are molecules that mediate T cell homing to lymph nodes through high endothelial venules (Masopust et al., 2001; Sallusto et al., 1999). Other markers include the altered glycosylation pattern on various cell surface glycoproteins, such as CD43 and CD45 (Galvan et al., 1998; Harrington et al., 2000). These different cell surface markers have since led to the idea that there are two distinct populations of memory cells T effector memory, (T_{EM}) and T central memory, (T_{CM}). The T_{EM} population are CCR7⁻ and CD62L⁻, and are present in the blood, spleen and non-lymphoid tissue while T_{CM} population are CCR7⁺ and CD62L⁺, and reside in the lymph nodes, spleen and blood but not in non-lymphoid tissue (Sallusto et al., 1999). Studies have shown that T_{EM} population tend to respond faster to antigen than the T_{CM} population, with respect to their ability to produce effector function (Masopust et al., 2001; Sallusto et al., 1999). Other studies show in contrast that CCR7 and CD62L do not correlate with effector functions of memory CD8 T cells. Both subsets are equally efficient at producing effector cytokines and becoming killer cells upon antigen recall (Unsoeld et al., 2002; Wherry et al., 2003).

1.8.2 Differentiation of memory CD8 T cells

Cell Extrinsic Factors

Majority of effector CD8 T cells upon effective clearance of virus die via apoptosis (Badovinac et al., 2002; Badovinac et al., 2004; Kaeck et al., 2002b). 5-10% survive the contraction phase and differentiate into long-lived memory

CD8 T cells. Recently studies have shown that a memory precursor pool may exist among the effector CD8 T cells, which are programmed to survive the contraction phase. These cells have been shown to express the cell surface interleukin 7 receptor alpha chain (IL-7R α) following LCMV infection and also by measuring mRNA levels in naïve, effector and memory CD8 T cells (Kaech et al., 2003). Kaech et al also showed that IL-7R α high memory CD8 T cells contained increased expression of anti-apoptotic molecules Bcl-2 and Bcl-X_L. In another study Madakamutil et al showed the expression of homotypic form of CD8, CD8 $\alpha\alpha$ also promoted the survival of CD8 memory T cell precursors. Here CD8 $\alpha\alpha$ was expressed on a subset of CD8 $\alpha\beta$ T cells during LCMV infection and in agreement with Kaech et al CD8 $\alpha\alpha$ expression correlated with increased IL-7R α and IL-15R β , which is also typically upregulated on memory CD8 T cells (Madakamutil et al., 2004). In a more recent study Lacombe et al have challenged the notion of IL-7R α expression demarcates the population of effector CD8 T cells destined to become long – lived memory cells. Here they show adoptive transfer of CD8 TCR transgenic T cells into a naïve host followed by peptide pulsed DC or peptide with adjuvant immunisation. No enrichment of IL-7R α expression was observed on effector CD8 T cells during the contraction phase (Lacombe et al., 2005). There are however striking contrasts in the downregulation of IL-7R α between the two models. In the LCMV model used by Kaech et al, IL-7R α levels were completely abrogated at the peak of the antigenic response, whereas Lacombe et al showed fewer effector CD8 T cells had down regulated IL-7R α expression. The degree to which IL-7R α expression is down regulated could be influenced by the

magnitude of the expansion phase. In the LCMV model the expansion phase is massive reaching over 20×10^6 antigen specific CD8 T cells while in the peptide pulsed DC model expansion phase is more modest at less than 10^6 . The massive expansion phase leads to the production of high IL-2 levels that are capable of abrogating IL-7R α expression on the LCMV specific effector CD8 T cells. To further survive and differentiate into memory T cells, the effector cells re-express IL-7R α , and this small percentage will subsequently survive the contraction phase. Due to the partial down regulation of IL-7R α in effector CD8 T cells in the model described by Lacombe et al; it is suggested that other factors besides IL-7 α expression participate in the fate of effector CD8 T cells, especially those destined to become memory cells.

A recent study conducted by Buentke et al showed that both IL-7 and IL-15 have the capacity to promote survival of effector cells to form memory, but only in circumstances that resulted in enhanced signalling through their respective receptors such as in a lymphopenic environment (Buentke et al., 2006). Under physiological conditions, where both IL-7 and IL-15 are subject to host cell competition and therefore likely to be limited in availability, the formation of memory cells was found to be strictly dependent on the expression of IL-7R. However both IL-7 and IL-15 acted in synergy to promote the formation of memory cells by directly limiting the contraction of effector cells following an immune response and re-expression of the IL-7R was a key checkpoint in the regulation of this process (Buentke et al., 2006).

Cell Intrinsic Factors

Recent studies have shown the transition from effector to memory CD8 T cells are associated with marked changes in gene expression (Kaech et al., 2002a). The molecular link between the programming of effector to memory CD8 T cells has been demonstrated by the identification of the tissue specific T-box transcription factors T-bet and eomesodermin. T-bet is responsible for controlling the lineage commitment in TH₁ cells (Szabo et al., 2000). CD4 T cells and NK cells from T-bet^{-/-} mice are defective in their ability to produce IFN γ . CD8 T cells from these mice can exhibit normal IFN γ induction and cytolytic function (Szabo et al., 2000; Szabo et al., 2002) implying a T-bet independent pathway for effector differentiation in CD8 T cells. Over-expression studies, however using dominant negative factors have shown that both T-bet and EOMES have co-operative or redundant functions regulating the genes encoding IFN γ and cytolytic molecules including perforin and granzyme B in CD8 T cells (Pearce et al., 2003). More recently studies have been conducted to ascertain whether EOMES is essential for T-bet independent control of cell-mediated immunity. EOMES^{-/-} mice (Russ et al., 2000) are embryonic lethal and so the regulation of effector function by these T-box factors were determined through the analysis of compound mutant *Eomes*^{+/-} *Tbx21*^{-/-} mice (Intlekofer et al., 2005). These mice have a substantial loss of NK and NKT cells and also a striking depletion in memory phenotype CD8 T cells with high CD122 expression. *EOMES*^{+/-} mice have small and detectable defect in memory CD8 T cells whereas *Tbx21*^{+/-} resembled wild type mice. Thus indicating that both the T-box factors contribute to the homeostasis of memory

CD8 T cells. *In vitro* activated CD8 T cells from *Eomes^{+/-}Tbx21^{-/-}* mice had defective induction of IFN γ in agreement with the dominant negative studies conducted previously (Pearce et al., 2003). Similarly there is a loss in mRNA levels of perforin in bone marrow NK cells. It would appear therefore that EOMES and T-bet cooperate in effector functions for cell mediated immunity.

1.8.3 Maintenance of Memory CD8 T cells

Memory CD8 T cells can persist in the absence of antigen and the role for cytokines IL-7 and IL-15 in the maintenance of memory CD8 T cells has been identified (Becker et al., 2002; Goldrath et al., 2002; Schluns and Lefrancois, 2003). Studies by Zhang et al showed memory phenotype CD8 T cells (CD44 High) selectively expressed high levels of CD122 (IL-2/IL-15R β), a receptor molecule shared by both the IL-2 and IL-15 receptors. IL-15 was shown to cause selective proliferation of CD44 high CD8 T cells (Zhang et al., 1998c). Another study demonstrated that the *in vivo* blockade of CD122, resulted in the inhibition of memory CD8 T cells, showing a role for IL-15 in memory CD8 T cell survival (Ku et al., 2000). The evidence for the role of IL-15 in memory CD8 T cell maintenance came from the generation of IL-15R α (Lodolce et al., 1998) chain or IL-15 (Kennedy et al., 2000) deficient mice. Analysis of these mice showed that memory CD8 T cells could be generated but the maintenance was affected as numbers appeared to decline over time. Maintenance of memory CD8 T cells was shown to be due to homeostatic proliferation. Experiments were conducted in which IL-15^{-/-} mice were infected with LCMV, and antigen

specific memory CD8 T cells were adoptively transferred into wild type IL-15 sufficient mice and regained the ability to proliferate (Becker et al., 2002).

In contrast to IL-15, IL-7 appears to be important for the survival rather than the proliferation of memory CD8 T cells (Kieper et al., 2002; Lodolce et al., 1998; Maraskovsky et al., 1997). Transgenic mice expressing IL-7 driven from the MHC class II promoter had increased numbers of memory CD8 T cells, however the turnover rate was that of normal cells (Kieper et al., 2002). As mentioned previously the expression of IL-7R α is down regulated on the majority of effector CD8 T cells generated during a viral infection. The subset that do survive and subsequently generate into memory cells are those that express elevated levels of IL-7R α (Kaech et al., 2003). Part of the survival may be due to the sustained expression of anti-apoptotic Bcl-2 induced by IL-7 (Maraskovsky et al., 1997).

1.9 Lck Inducible System

The Lck^{-/-} mice have very few T cells in the periphery with severe phenotypic abnormalities. To evaluate the role of Lck in peripheral T cell responses a transgenic system was developed in the laboratory to express Lck in an inducible manner (**Figure 1.6**). This was achieved using a transgenic mouse expressing a tetracycline-inducible transactivator (rtTA) (Furth et al., 1994; Gossen and Bujard, 1992; Gossen et al., 1995), under the control of a human CD2-regulatory element, which is constitutively expressed in a tissue specific manner in T cells and at a low level in B cells (Zhumabekov et al., 1995).

These mice were crossed with transgenic mice expressing an *lck* transgene under the control of a tetracycline –responsive/minimal CMV promoter, and both lines were on the *Lck*^{-/-} background (Legname et al., 2000). *Lck* expression is thus only detected and hence controlled after administration of the tetracycline derivative, doxycycline (dox) in the feed of the mice from before birth to at least 6 weeks of age (**Figure 1.5**). The induction of the *lck* transgene overcomes the lack of endogenous *Lck* expression and restores thymopoiesis to that of normal levels (Legname et al., 2000). This system is used to keep *Lck* expression on during the important stages of thymocyte development (*Lck*^{ind} ON) via the administration of dox in the feed of the mice, and once a mature peripheral T cell repertoire is formed, *Lck* expression can be switched off by the removal of dox from the feed (*Lck*^{ind} OFF) and so we are able to address questions relating to the role of *Lck* in peripheral T cell responses.

Naïve T cell Survival

Using the *Lck*^{ind} model, the requirement for TCR mediated signals in naïve T cell survival were determined. It was suggested that TCR mediated survival signals may be mediated through *Lck* and /or *Fyn* in a similar manner to TCR signals being required for T cell differentiation and activation. Upon withdrawal of dox *Lck* transcription is terminated, and the residual *Lck* protein is undetectable after 7 days (Seddon et al., 2000). Switching *Lck* expression off also results in cessation of thymopoiesis (Seddon and Zamoyska, 2002b). This model is useful in measuring the lifespan of naïve T cells. The loss of *Lck* had no effect on the survival of peripheral T cells for many months. However, when

the Lck^{ind} mice were crossed onto an Lck^{-/-}Fyn^{-/-} background and Lck was switched off, so that neither Lck nor Fyn was expressed naïve T cell survival was affected. The half – life of the peripheral T cells was approximately 26 days for CD4 T cells and 18 days for the CD8 T cell compartment. All TCRζ chain phosphorylation was lost and a significant decline in CD5 expression was also observed, consistent with a loss in stimulation through the TCR (Seddon and Zamoyska, 2002b). The data shows that Lck or Fyn expression is required for long-term naïve T cell survival. Alongside the requirement for Src kinases Lck or Fyn for naïve T cell survival, IL-7 was also shown to contribute to the long half-life of the peripheral T cells. When Lck^{ind}/lck^{-/-}fyn^{-/-} off dox mice were treated with blocking IL-7R antibody the half-life was further reduced to 2 weeks (Seddon and Zamoyska, 2002b). Naïve T cell survival therefore requires the synergistic signals mediated by cytokine signalling through IL-7R and TCR/MHC interactions.

Homeostatic Proliferation

Signals which drive the proliferation of naïve T cells under the conditions of lymphopenia, have also been studied using the Lck^{ind} model. It was originally thought that the signals required for T cell survival might be the same signals required for lymphopenia-induced proliferation. The Lck^{ind} system was used to determine what factors were involved in lymphopenia-induced proliferation. When T cells from Lck^{ind} mice were transferred into a mildly lymphopenic host (lck^{-/-}) proliferation was not observed these cells showed a complete dependence on Lck for proliferation, while the absence of Fyn had no effect

(Seddon et al., 2000; Seddon et al., 2003). By contrast upon transfer of naïve T cells into more severe lymphopenic hosts such as Rag1^{-/-} or Lck^{-/-}Fyn^{-/-}, naïve T cells were able to proliferate even in the absence of Lck. Similarly when Lck^{ind} CD4 T cells were transferred into MHC Class II deficient host, in the absence of the ligand required to stimulate the TCR, naïve T cell proliferation was unaffected showing that lymphopenia induced proliferation was TCR/MHC independent. This TCR independent proliferation was shown to be mediated by IL-7R signalling (Seddon et al., 2003). Together the data show a role for both TCR and cytokine mediated signals for lymphopenia-induced proliferation. Importantly the TCR signalling component was shown to be essentially dependent on signalling through Lck but not Fyn.

1.10 Thesis Aims

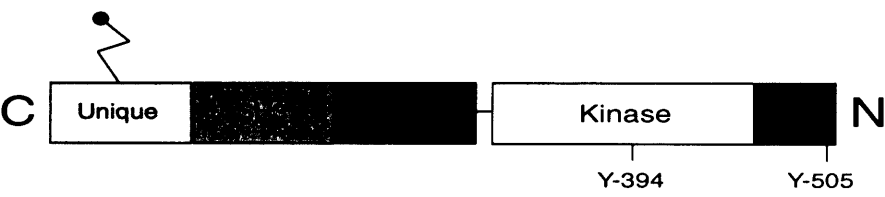
Signalling through the TCR regulates the differentiation and function of T cells throughout their life span. Using the Lck inducible model (Lck^{ind}) the importance of Lck signalling for thymocyte development and homeostatic maintenance of the naïve population has been addressed (Basson and Zamoyska, 2000; Seddon and Zamoyska, 2003).. Yet little is known about the exact signalling mechanisms involved in the generation of effector and memory CD8 T cells and eliciting effector functions. Since Lck is one of the first signalling molecules to be activated following TCR interaction with pMHC molecules, we examined the response of CD8 T cells, which encounter antigen in the low level or complete absence of Lck expression in comparison to wild type cells. Hence the aims of my thesis are to examine:

-
- The role of Lck in the generation of effector CD8 T cell function *in vitro*.
 - The role of Lck in the generation of effector CD8 T cells *in vivo*.
 - The role of Lck in the generation and maintenance of memory CD8 T cells.

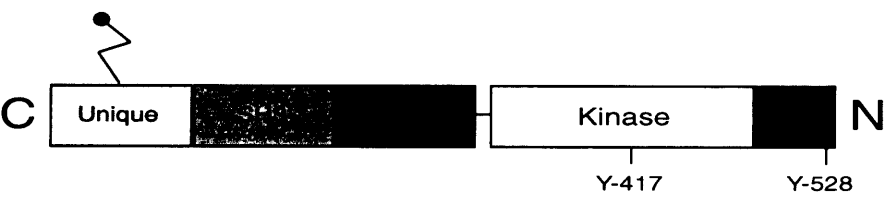
Figure 1.1 Structure of Lck and Fyn

Lck and Fyn are closely related in structure and have some overlapping functions. They share most homology through the kinase domain and also have significant homology through the SH2 and SH3 domains. The unique domains however show no homology and have unique modifications that are essential for their targeting to the plasma membrane and lipid rafts.

Lck



Homology	0%	71%	75%	81%
Identity		51%	58%	67%



Fyn

Figure 1.2 TCR Signal Transduction Pathways Downstream of Lck

Diagram to show the key early TCR signal transduction pathways involved upon interaction of the TCR with antigen/MHC. ITAMs within the CD3 complex are phosphorylated by the Src kinases Lck and Fyn. This leads to the recruitment and phosphorylation of the Syk kinase, ZAP-70 by Lck. This leads to the subsequent activation of other downstream signalling molecules via various pathways leading to the transcription of target genes.

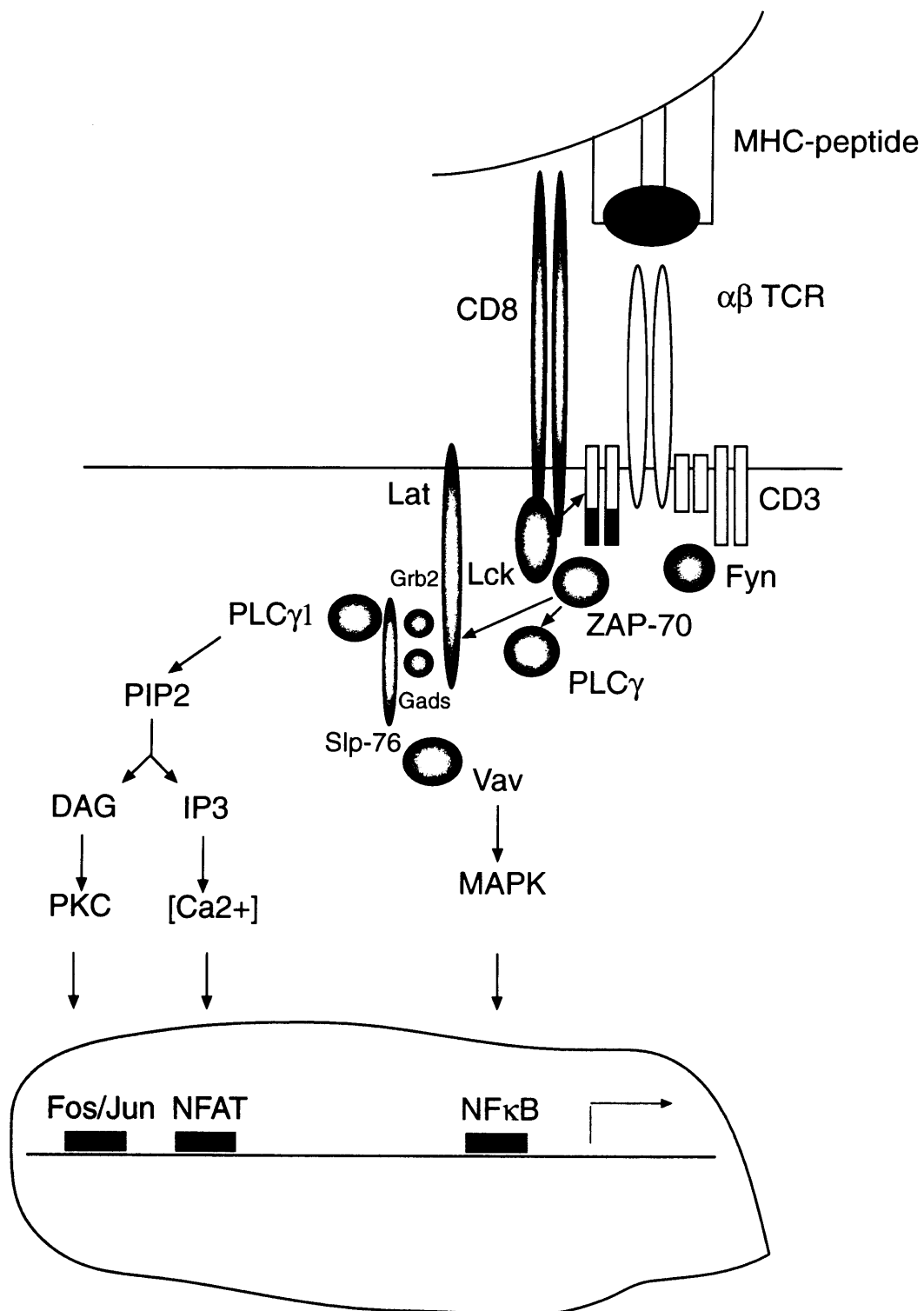


Figure 1.3 JAK/STAT Signal Transduction Pathway

A schematic representation of the JAK/STAT signal transduction pathways; which are activated upon engagement of cytokines (IL-2 and IFN γ) and their receptors (IL-2R and IFN γ).

Figure 1.4 - Role of Lck in T-cell Development

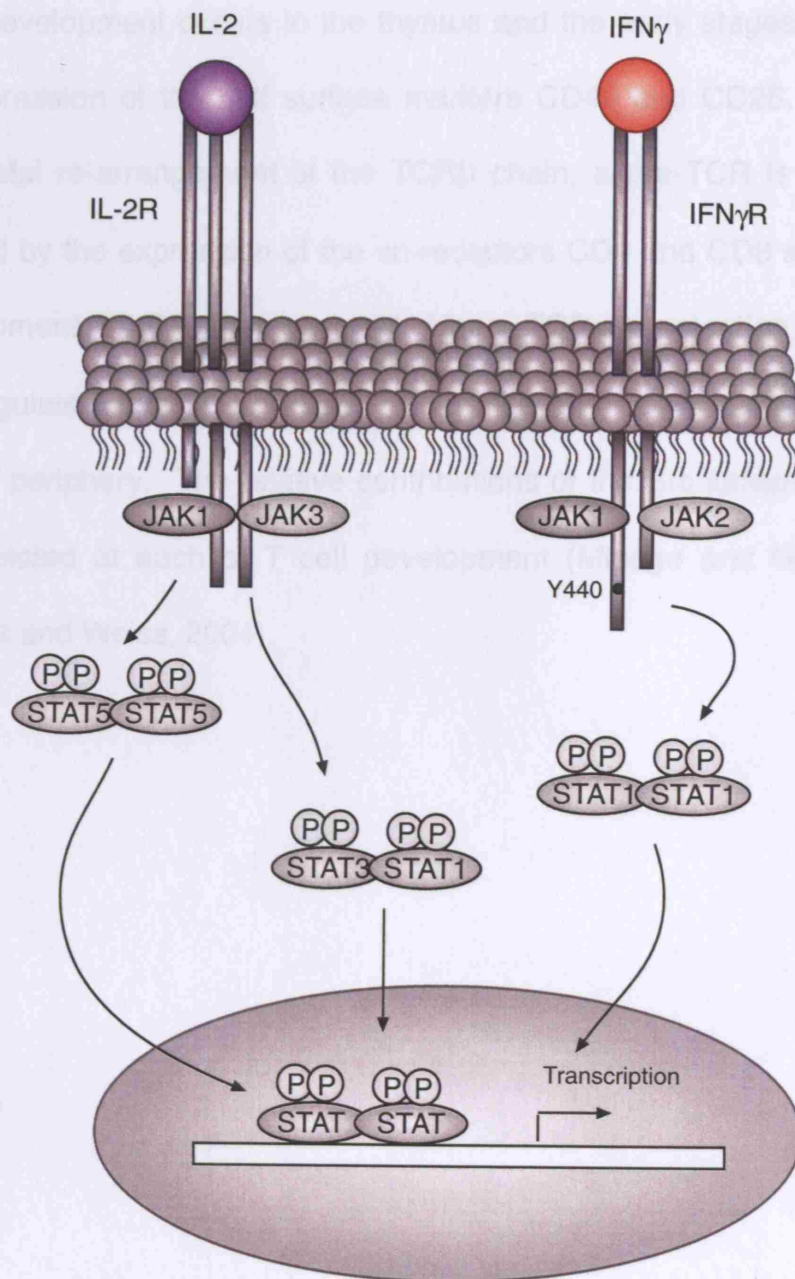


Figure 1.4 Role of Lck in T cell Development

T cell development occurs in the thymus and the early stages are defined by the expression of the self surface markers CD44 and CD25. Following the successful re-arrangement of the TCR β chain, a pre-TCR is formed; this is followed by the expression of the co-receptors CD4 and CD8 and leads to the development of DP thymocytes. Upon TCR $\alpha\beta$ selection, the DP cells downregulate either CD4 or CD8. The mature SP T cells subsequently migrate into the periphery. The relative contributions of the Src kinases, Lck and Fyn are depicted at each of T cell development (Miosge and Goodnow, 2005; Palacios and Weiss, 2004).

Figure 1.5 Generation of Memory CD8 T cells

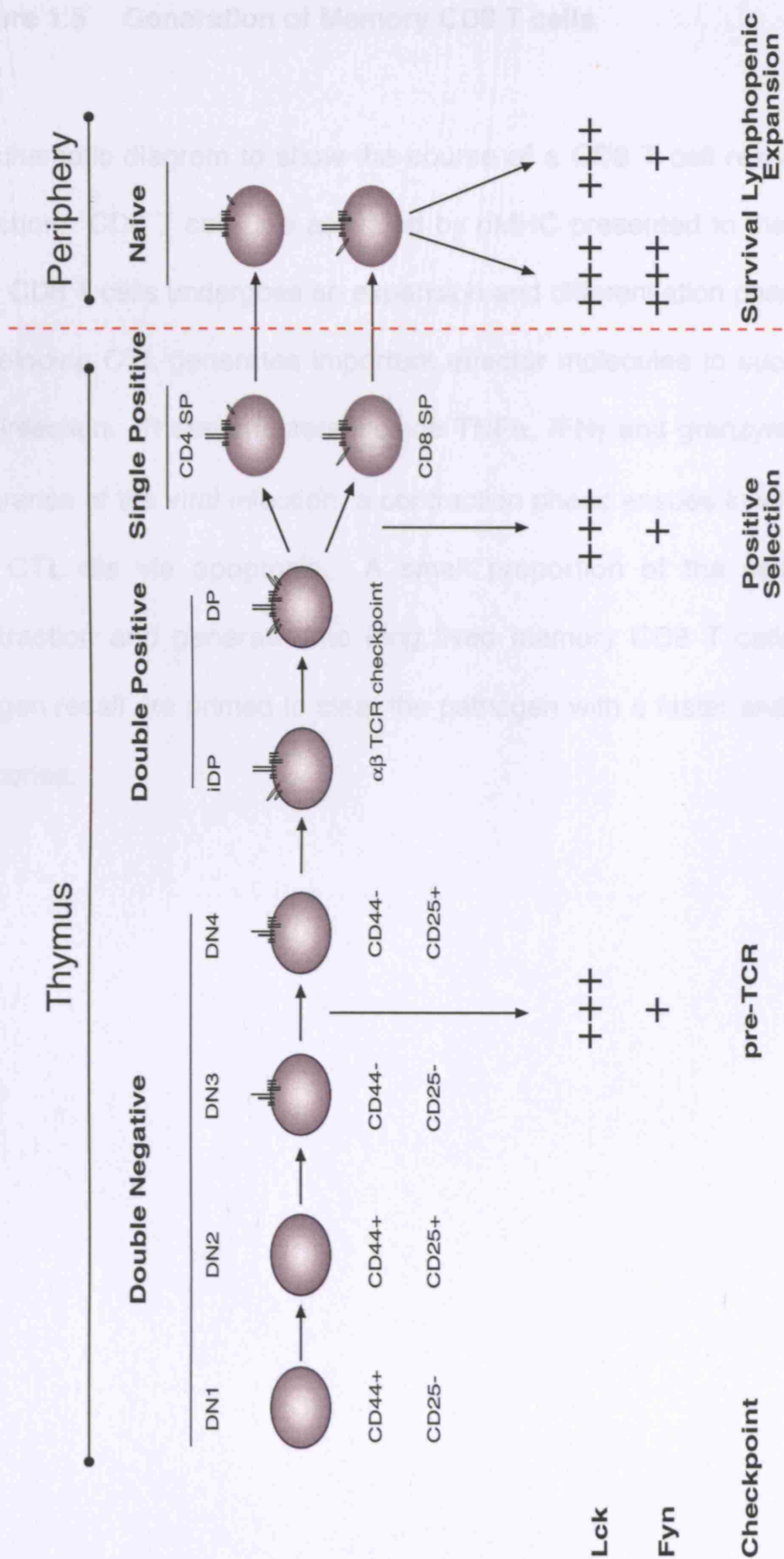


Figure 1.5 Generation of Memory CD8 T cells

A schematic diagram to show the course of a CD8 T cell response to a viral infection. CD8 T cells are activated by pMHC presented to them by an APC. The CD8 T cells undergoes an expansion and differentiation phase in which the developing CTL generates important effector molecules to successfully clear the infection. These effectors include $\text{TNF}\alpha$, $\text{IFN}\gamma$ and granzymes. Following clearance of the viral infection, a contraction phase ensues in which 90-95% of the CTL die via apoptosis. A small proportion of the cells survive the contraction and generate into long lived memory CD8 T cells, which upon antigen recall are primed to clear the pathogen with a faster and more efficient response.

Figure 1.6 The Lok Inducible System

Diagram to show the lof^2 transgene is expressed upon administration of doxycycline in the form of the mice. The reverse tetracycline transactivator fusion protein RTA is expressed under the control of the human CD2 promoter. When the RTA is combined with doxycycline together they bind the tetracycline response element in the minimal pCMV promoter, which then drives the expression of the transgene.

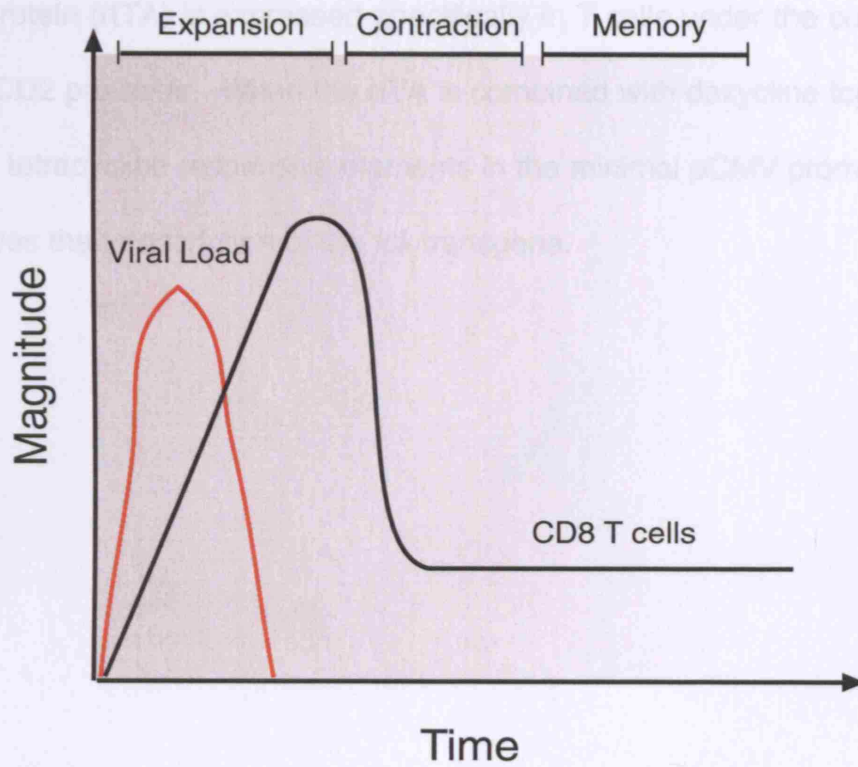


Figure 1.6 The Lck Inducible System

Diagram to show the *lck* transgene is expressed upon administration of doxycycline in the feed of the mice. The reverse tetracycline transactivator fusion protein (rtTA) is expressed specifically in T cells under the control of the human CD2 promoter. When the rtTA is combined with doxycycline together they bind the tetracycline responsive elements in the minimal pCMV promoter, which then drives the transcription of the *lck* transgene.

2: Materials and Methods

2.1 Solutions

Handling Media

Air Buffered (AB) IMDM/2% HI-FCS

Culture Media

IMDM (Sigma, UK)

10% Heat Inactivated – FCS (Gibco BRL, UK)

100U/ml Penicillin (Sigma, UK)

100µg/ml Streptomycin (Sigma, UK)

2x10⁻³M L-Glutamine (Sigma, UK)

5x10⁻⁵M β-mercaptoethanol (Sigma, UK)

FACS Buffer

PBS/0.5% Sodium Azide/0.5% BSA

Immunisation Media

Dulbecco's PBS (Invitrogen, UK)

10 X Permeabilisation Buffer

1% NP40 (Sigma,UK)

50mM Tris, pH 7.6

150mM NaCl

2.2 Mice

Lck^{-/-} mice harbouring the Lck-1 transgene controlled by the tetO/CMV promoter were crossed with Lck^{-/-} mice containing the rtTA under the control of the huCD2 LCR generating Lck^{ind} mice (Legname et al., 2000). The Lck^{ind} line was also crossed onto a class I MHC-restricted F5 TCR transgenic line (Mamalaki et al., 1992) to generate monoclonal CD8 T cells, on a Rag-1^{-/-} background (Mombaerts et al., 1992). Lck expression was maintained by administering 1mg/g dox (Sigma, UK) in the feed of the Lck^{ind} ON mice. To turn off Lck expression mice were taken off dox feed 7 days prior to experiments as it has previously been shown that Lck expression is absent by this time (Lck^{ind} OFF) (Seddon et al., 2000). The genotype of all mice was determined by a combination of PCR and FACS analysis of the peripheral blood lymphocytes. All mice were bred and housed under SPF conditions under home office regulations at the NIMR animal facility. The B6.MRL-^{Fas^{lpr}}/J mice have been previously described (Andrews et al., 1978); and were a kind gift from Prof. Marina Botto, *Imperial College*.

Strain	Feed Type	References
C57BL/6	Normal	-
C57BL/6 CD45.1	Normal	-
C57BL/10Rag-1 ^{-/-}	Normal	(Mombaerts et al., 1992)
Lck-1het rtTA Chet Lck ^{-/-}	1mg Dox	(Legname et al., 2000)
F5 het Rag-1 ^{-/-}	Normal	(Mamalaki et al., 1992)
F5 het Rag-1 ^{-/-} CD45.1/CD45.2	Normal	-

Lck-1het rtTA Chet F5 het Lck ^{-/-}	1mg Dox	-
B6.MRL ^{-Fas^{lpr}/J}	Normal	(Andrews et al., 1978)
Lck-1homRag1 ^{-/-} F5homLck1 ^{-/-}	Normal	-
B6.129P2- <i>Il2^{tm1Hor}</i>	Normal	(Schorle et al., 1991)

Table 2.1 Mice used in study

2.2.1 Radiation Bone Marrow Chimeras

Lck-1homRag1^{-/-}F5homLck1^{-/-}mice were irradiated for 5 minutes with 500 Rads (Caesium source) and were left overnight before transferring bone marrow cells. The mice were treated with irradiated water supplemented with 0.02% v/v Baytril® (Sigma, UK) for 5 weeks. Bone marrow from the back legs (Tibia and Fibia) of C57BL/6 CD45.1 mice was isolated using a fine gauge needle and 1 ml syringe to flush handling media through the bone. Bone marrow cells were washed once by centrifugation at 4° C. Bone marrow cells were incubated with Thy1.2 antibody and mature T cells were depleted from the bone marrow cells using the Dynal bead depletion strategy detailed in section 2.9.1. 10 x10⁶ bone marrow cells were transferred i.v. via the tail vein into the irradiated mice. The mice were left for 6-8 weeks to enable reconstitution of lymphoid cells. These mice were used in experiments described in Chapter 4 Section 4.5.

2.3 Cell Isolation

The axillary brachial, cervical, inguinal and mesenteric lymph nodes were harvested from age matched mice. Spleen and thymus were also taken in some experiments. Single cell suspensions were prepared by gently teasing the organs through sterile gauze in AB-IMDM/2% FCS. Cells were washed and counted using Casy-1 automated cell counter (Scharfe System, Germany).

2.4 *In vitro* T cell Stimulation

F5 CD8 T cells were stimulated in an antigen specific manner using soluble NP68 (see 2.4.1). 2×10^5 LN derived cells (>90% T cells) were plated in a 96 well round bottomed plate (Nunc, Denmark) in culture media. The cells were cultured with NP68 at 37°C/5%CO₂ for specific time points depending on the experimental conditions and analysed.

2.4.1 Peptide

The peptide antigen influenza virus nuclear protein 68, NP68 (Ala, Ser, Glu, Asn, Met, Asp, Ala, Met) is recognised by the F5 TCR transgenic mouse line in the context of H-2D^b (Mamalaki et al., 1992; Townsend et al., 1986). The peptide was synthesised by the Division of Protein Structure at the NIMR and stored at -20°.

2.4.2 Doxycycline

For *in vitro* cultures, 1 µg/ml Dox solution was added at the start of the culture to Lck^{ind} ON T cell suspension to maintain expression of the Lck transgene throughout the culture period.

2.5 4 – Colour Flow Cytometry

Analysis of *in vitro* and *ex vivo* cell surface and intracellular protein expression was carried out using 4-colour flow cytometry. 1×10^6 T cells were stained with directly conjugated fluorescent antibodies or biotinylated antibodies. For biotinylated antibodies were required the secondary antibody streptavidin PerCP was used as a second layer. Directly conjugated antibodies were diluted as appropriate in ice cold FACS buffer. Cells were incubated for 30 minutes on ice with the antibody cocktail. Cells were incubated with a secondary antibody for 10 minutes on ice, with an intermittent wash step. Cells were washed and resuspended in 100 µl of FACS buffer prior to analysis on the flow cytometer (FACScalibur, BD). Data analysis was performed using FloJo software platform (Tree star, USA).

Intracellular staining

Detection of intracellular cytokines in activated T cells was carried out by incubating cells with 0.5 µg/ml PdbU (Sigma, UK), 0.5 µg/ml Ionomycin (Sigma, UK) and 1 µg/ml Brefeldin A (Sigma, UK) for 4 hours at 37°C/5%CO₂. Alternatively in some experiments T cells which were activated *in vivo* were incubated with 1 µM NP68 in culture media overnight and subsequently treated

with 1µg/ml Brefeldin A at 37°C/5%CO₂. Cells were washed and stained for cell surface markers, and then fixed with 3% paraformaldehyde for 20 minutes on ice. The cells were washed and resuspended in 100µl of permeabilisation buffer for exactly 3 minutes. 100µl of FACS buffer was added to the cells to stop the reaction. The cells were resuspended in FACS buffer containing the fluorescently labelled intracellular marker or cytokine and incubated for 30 minutes on ice. The cells were washed and resuspended in 100µl of FACS buffer and the samples were acquired on the FACScalibur. For Granzyme B detection of *in vitro* activated T cells, the cells were not incubated with the PdbU/Ionomycin/Brefeldin A cocktail.

Antibodies used in Flow Cytometry

Marker	Clone	Fluorescent Conjugate	Supplier
TCRβ	H57-597	FITC/APC/Biotin	BD
CD4	RM 4-5	FITC/PE/PerCP/APC	BD & eBioscience
CD5	53-7.3	FITC/APC/Biotin	BD
CD8α	53-6.7	PE/APC/PerCP	BD
CD8β	53-5.8	FITC	eBioscience
CD16/CD32			
Fcγ III/II	2.4G2	Purified	BD
CD25	7D4	FITC/APC	BD
CD25	PC61	PE	BD
CD43	1B11	PE	BD
CD44	IM7	PE/APC/Biotin	BD

CD45.1	A20	PE	BD
CD45RB	23G2	PE	BD
CD62L	MEL-14	PE	BD
CD69	H1.2F3	FITC/PE/Biotin	BD
CD95L	MFL3	Biotin	BD
CD122	5H4	FITC	BD
IFN γ	XMG1.2	FITC/PE	BD
TNF α	6401.1111	PE	BD
Granzyme B	GB12	PE	Caltag
Isotype control	MOPC-21	PE	Caltag

Table 2.2 Cell surface and intracellular antibodies used for FACS analysis.

2.6 Measurement of *In vitro* Cytotoxicity

2.6.1 Generating CTLs

Spleen was harvested from F5 mice and cell suspensions were prepared and activated with NP68. To generate effector cells F5 WT cells were activated with 100nM NP68, and the Lck^{ind} ON and OFF cells were incubated with 1 μ M NP68 in culture media (as determined in Chapter 3 section 3.7). Cultures were grown in upright 50ml culture flasks (Becton Dickson, France), in a 10ml at 3x10⁶ cells/ml for 72 hours at 37°C/5%CO₂. CTLs were washed and counted using a haemocytometer and trypan blue staining ensuring only live blast cells were counted. Cells were resuspended at 1x10⁷ cells/ml.

2.6.2 Maintaining EL-4 thymoma cell line

The target EL-4 thymoma cell line was maintained in culture media and split at ~80% confluence. The day before the assay the cells were split to ensure they were in log phase of growth. EL-4 cells were washed, and resuspended at 6×10^6 cells/ml in serum free AB-IMDM. 2x 500 μ l aliquots were taken, one aliquot was pulsed with 10 μ M NP68, and the other aliquot remained un-pulsed as a control. Both aliquots were labelled with 1.85Mbq ^{51}Cr , sodium Chromate (Amersham Bioscience, UK). The cells were incubated for 90 minutes at 37°C/5%CO₂. EL-4 cells were washed twice and resuspended at 1×10^5 /ml.

2.6.3 ^{51}Cr Release Assay

Effector cells generated in 2.6.1 were added to 96 well round bottomed plates in triplicate at the top effector to target ration, and serially diluted 8 times 1 in 3. To the effectors, 1×10^4 ^{51}Cr labelled EL-4 cells were added to each well, giving the correct effector to target ratio. To measure non-specific or spontaneous lysis of target cells, ^{51}Cr labelled un-pulsed EL-4's cells were added to effectors as a control. To measure maximum ^{51}Cr release 1% Triton-X was added to peptide pulsed EL-4 targets. The plate was spun down for 1 minute at 1000rpm, and incubated for 4 hours at 37°C/5%CO₂. 25 μ l supernatant was harvested, and spotted onto a printed filter mat (Wallac, Finland). Filter mats were dried in a microwave oven for 1 minute. The dried mat was immersed in liquid scintillant (Wallac, Finland). ^{51}Cr release was measured using a 1205 automated β -plate counter (Wallac, Finland).

2.6.3 Calculation for ^{51}Cr Specific Release

NP68 specific lysis of EL-4 target cells was measured using the following calculation:

$$\frac{(\text{Experimental} - \text{Spontaneous Release}) \times 100}{(\text{Maximum} - \text{Spontaneous Release})} = \% \text{ Specific } ^{51}\text{Cr} \text{ release}$$

2.7 Measurement of IL-2 production by F5 CD8 T cells

CTLL-2 cells (ATCC cat no. TIB 214) were initially derived as a tumour specific CTL clone capable of long term growth *in vitro*, when maintained in media containing IL-2 (Deng and Podack, 1993; Gillis et al., 1978; Gillis and Smith, 1977; Nunez et al., 1990). In this bioassay cell culture media is harvested from *in vitro* activated T cells to measure IL-2 production. 50 μl of supernatant from each sample was removed and cultured with 5×10^3 CTLL-2 cells (ATCC cat no. TIB 214) for 24 hours at 37°C/5%CO₂. After 24 hours 10 μl of Alamar blue dye (Biosource, UK) was added and incubated overnight at 37°C/5%CO₂. Colour change in the dye was measured using a Luminescence Spectrometer LS50B (Perkin Elmer, UK) at 590nm. Data was analysed using FL WinLab software (Perkin, Elmer).

2.8 *In vivo* Cytotoxicity Assay

2.8.1 Generation of A/NT/60-68 Flu specific effector F5 CD8 T cells

LN cells were isolated from F5 mice and cell suspensions were made. Cells were resuspended in immunisation media 3×10^6 cells /150 μ l per mouse. A/NT/60-68 influenza virus stock is at 2000 haemagglutination (HA) units/ml and was diluted 1 in 5 in immunisation media. The flu virus was added to the F5 CD8 T cells at 20HA units per mouse in a 50 μ l volume. The optimal dose of virus was calculated prior to the experiments (Chapter 4 section 4.2). 200 μ l total volume of F5 CD8 T cells and flu were transferred i.v. via the tail vein into C57BL/10 Rag1^{-/-} (Rag1^{-/-}) mice immunisation media. Mice were left for 7 or 35 days to measure generation of effector and memory F5 CD8 T cells respectively.

2.8.2 Preparation of Target Cells

LNs and spleen were harvested from C57BL/6 CD45.1 and in some experiments B6.MRL-*Fas*^{pr/J} mice. Cell suspensions were made as described above. The cells were divided into four groups and resuspended in 10mls of culture media and each population was pulsed with different concentrations of NP68 for 2 hours at 37°C/5%CO₂ (**Table 2.3**). In some experiments target cells were divided into two groups where one population was pulsed with the highest peptide and the other population was left un-pulsed. The cells were washed and resuspended at 2×10^7 cells/ml in warm PBS. CFSE (Sigma, UK) solutions were made up in warm PBS at 2X the final concentration (**Table 2.3**). To each

group of cells, equal volumes of CFSE solution was added gently giving final concentrations as listed. Cells were incubated at room temperature for 10 minutes, washed twice and resuspended in immunisation media. Each population of cells was counted and mixed to a ratio of 1.4×10^6 total target cells in 200 μ l final volume and injected i.v. per mouse. After 18 hours the LN and spleen were harvested from recipient mice. Target cells were identified from the previously transferred F5 CD8 T cells based on their expression of the CD45.1 and CFSE incorporation.

Group	[NP68]	[CFSE]
1	0 Peptide	1.25 μ M
2	2.5 μ M	0.25 μ M
3	2.5 $\times 10^{-3}$ μ M	0.125 μ M
4	2.5 $\times 10^{-6}$ μ M	None/0.025 μ M

Table 2.3 CFSE and peptide concentrations used to label target cells.

2.8.3 Calculation of Specific Lysis

To calculate the NP68 specific lysis of the target cells the formula described in **Figure 2.1** was used. In a given mouse specific lysis of target cells was measured by normalising the percentage of each peptide pulsed peak to the un-pulsed (0 Peptide) control where the 0 peptide peak was considered as 100%. Each of the peptide pulsed peaks were expressed as a percentage of the un-pulsed control. In the experimental groups, each peak was also normalised relative to the un-pulsed peak, which was also expressed as 100%.

Each normalised experimental peak percentage was then expressed as a percentage of the control peaks for the relevant peptide concentration (**Figure 2.1**).

2.9 Cell Purification

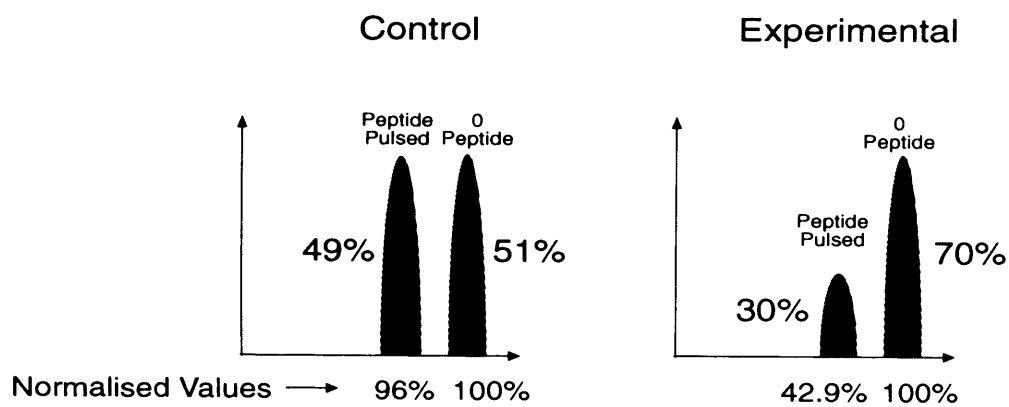
2.9.1 Dynal Bead Depletions

For negative depletion, cell suspensions were incubated with a cocktail of biotinylated antibodies on ice for 30 minutes (**Table 2.4**) at saturating concentrations in handling media (1ml/10⁸ cells). Samples were washed and resuspended in 4mls PBS. Handling media was not used as it contains biotin and would interfere with the dynal bead binding reaction. Dynal beads (M280, Dynal, Norway) were added at a 1:1 ratio of beads to cells for 30 minutes at 4°C with constant rotation. Cells bound to the dynal beads (negative fraction) were removed using a magnetic particle concentrator (Dynal, Norway). This step was repeated twice with an intermittent wash step to remove excess beads. Cells were resuspended in handling media for the subsequent step and counted. The extent of depletion was determined by flow cytometry.

Marker	Clone	Conjugate	Supplier
CD8 α	53-6.7	Biotin	BD
CD45R/B220	RA3-6B2	Biotin	BD
CD11b/Mac-1	M1/70	Biotin	BD
I-A ^b	25-9-17	Biotin	BD
CD49d	DX-5	Biotin	BD
CD90.2/Thy1.2	53-2.1	Biotin	BD

Table 2.4 Antibodies used for Dynal bead depletion

Figure 2.1 Calculation: NP68 specific lysis of target cells



$$\% \text{ Target Cells Remaining} = \frac{42.9 \text{ (Experimental)} \times 100}{96 \text{ (Control)}} = 44.7\%$$

$$\% \text{ NP68 Specific Lysis of Target Cells} = 100 - 44.7 = 55.3\%$$

Chapter 3: The Role of Lck in CD8 T cells and generation of effector function *in vitro*

3.1 Introduction

Signalling through the TCR regulates the differentiation and function of T cells throughout their lifespan. The most proximal signalling molecules to be activated upon TCR engagement with pMHC molecules are the Src family kinases Lck and Fyn (Zamoyska et al., 2003). Two components are thought to influence the generation of cytotoxic CD8 T cells, modulation of gene expression and re-distribution of signalling molecules downstream of the TCR.

Various studies have been conducted to understand how CTL are generated. Early work showed that in naive CD8 T cells few CD8 molecules associated with Lck and that Lck had homogenous distribution inside the cell. However, subsequent activation of the T cell induced, co-localisation of Lck and CD8 co-receptor in the plasma membrane. This suggests that there is more efficient organisation of signalling molecules in effector cells by allowing Lck to be in close proximity to the TCR (Bachmann et al., 1999b; Veillette et al., 1988). Recently studies have been carried out to analyse the molecular events that take place during CTL differentiation. Gene expression analysis showed mRNA levels of genes encoding signalling molecules including Lck, Fyn, CD45 phosphatase, grb-2, LIME and PEP phosphatases were elevated in effector CD8 T cells compared to naïve cells (Kaeck et al., 2002a).

However little is known about how the lack of Lck expression influences the response of primary T cells upon activation. The ability of Lck^{-/-} mice to resist viral infection has been examined, and it was shown that the generation of CTL and production of IgG antibodies was compromised and the mice failed to clear viral infection (LCMV and VSV-recombinant vaccinia virus) (Molina et al., 1993). Existing models are inadequate to fully understand the role of Lck in the generation of CTL because Lck^{-/-} mice have reduced numbers of T cells in the periphery. Therefore an insufficient response may have manifested due to defects in T cell development, which could have also induced alterations in the T cell repertoire (Molina et al., 1992).

To evaluate a role for Lck in peripheral T cell responses, a transgenic system was developed in our laboratory to express Lck in an inducible manner (**described in Chapter 1 section 1.9**). One feature of the Lck^{ind} system is that the expression levels of Lck in the peripheral T cell compartment is 10 – fold reduced compared with WT mice (Legname et al., 2000; Seddon et al., 2000; Seddon and Zamoyska, 2002b). Therefore we have a system in which we can study the role of peripheral T cell responses both in the partial and complete absence of Lck expression.

Previously studies have shown that the expression of Lck is required for the proliferation of activated CD4 T cells in response to stimulation by anti-CD3 antibody (Lovatt et al., 2006; Seddon et al., 2000). The level of Lck expression also determined the extent to which naïve CD4 T cells were triggered and

upregulated early activation markers CD69 and CD25 (Lovatt et al., 2006; Seddon et al., 2000).

However the role of Lck signalling in the activation of primary antigen specific CD8 T cells has not been addressed. To investigate the differentiation of effector CD8 T cells we used a class I MHC restricted F5 TCR transgenic mouse (Mamalaki et al., 1992), which was bred to the Lck^{ind} transgenic line (F5 Lck^{ind}). The F5 Lck^{ind} mice therefore have a monoclonal CD8 T cell repertoire that recognises a nonamer peptide NP68 of the influenza virus A/NT/60-68 in association with the MHC class I molecule H-2D^b (Mamalaki et al., 1992; Smyth et al., 2002). In this Chapter I will describe the F5 Lck^{ind} mice, which were used to study the activation of CD8 T cells and the generation of CTLs *in vitro*. We showed in the partial and complete absence of Lck expression naïve F5 CD8 T cells were inefficiently triggered and CTL generated in the complete absence of Lck expression displayed reduced production of effector cytokines and were unable to elicit cytolytic function.

3.2 Lck is required for normal T cell development in F5 mice

Polyclonal Lck^{ind} mice and the F5 TCR transgenic mice have been previously described and extensively characterised (Legname et al., 2000; Mamalaki et al., 1992; Seddon et al., 2000; Smyth et al., 2002). We analysed the thymi from F5 Lck^{ind} mice to confirm normal thymocyte development for this system. The F5 Lck^{ind} mice were crossed with Rag1^{-/-} mice to ensure exclusive differentiation to the CD8 SP lineage. **Figure 3.1A** shows CD4 versus CD8 FACS plots from the

thymi of F5 mice. We observed normal thymocyte development in the F5 WT mice, with no SP CD4 T cells, whereas F5 Rag1^{-/-} Lck^{-/-} mice showed an arrest in thymocyte development at the DN stage. These observations are in line with previous work carried out in polyclonal Lck^{-/-} mice (Anderson et al., 1993; Molina et al., 1992). A more detailed dissection of the DN subsets showed that T cell development was arrested between the DN3-DN4 transition at the stage of TCR β selection (Anderson et al., 1993). The CD44 and CD25 FACS profiles in **Figure 3.1C & D** also showed that F5 Rag1^{-/-} Lck^{-/-} and F5 Lck^{ind} OFF mice have a block in thymocyte differentiation at the DN3 stage with very few thymocytes progressing into the DN4 stage. Upon expression of the *lck* transgene in the F5 Lck^{ind} ON mice we were able to restore thymopoiesis comparably to that of F5 WT mice (**Figure 3.1A and B**). The removal of dox from the F5 Lck^{ind} ON mice leads to the complete cessation of Lck expression and disruption of further thymocyte development as previously shown (Seddon et al., 2000). Interestingly, a small accumulation of thymocytes in the DN3 subset of the F5 Lck^{ind} ON mice was observed, which was absent in F5 WT mice. The most likely explanation for this partial DN3 block is that some cells may not have expressed the transgene at this stage and so their further development was blocked. Also we observed a greater number of DP cells in the F5 Lck^{-/-} mice compared with F5 Lck^{ind} mice recently taken off dox (**Figure 3.1B**). In the absence of Lck expression a small proportion of developing thymocytes will differentiate into DP cells mediated by signals through Fyn, as shown by Lck^{-/-} Fyn^{-/-} mice which have a complete block at DN3-DN4 (Groves et al., 1996). The F5 Lck^{-/-} and F5 Lck^{ind} OFF mice were not age matched which could account for

the difference in DP numbers, alternatively the acute cessation of Lck may not have allowed sufficient time for Fyn-mediated selection to take place in F5 Lck^{ind} OFF mice, thus accounting for the reduced DP numbers. Importantly in the F5 Lck^{ind} OFF mice we saw a substantial population of SP CD8 T cells, which had not yet exited the thymus into the periphery (**Fig 3.1A & B**).

The focus of the following study is to evaluate the role of Lck in peripheral CD8 T cell response therefore we characterised the peripheral T cells in the F5 mice, to ensure we had generated normal F5 CD8 T cells.

3.3 Lck affects the number of CD8 T cells in the periphery of F5 mice

Analysis of F5 CD8 T cells from the LN and spleen showed that the cells that were generated were of normal phenotype. Cell surface expression of a variety of activation markers (data not shown) including CD69, CD25 and CD44 demonstrated that there were no differences between F5 WT and F5 Lck^{ind} CD8 T cells in the periphery and therefore both had a naïve phenotype. We also measured the cell surface expression of TCR (data not shown), CD8 and CD5. CD8 expression was equivalent between the different groups of F5 mice despite the different levels of Lck expression (**Figure 3.2A**). In the polyclonal Lck^{ind} mice CD4 levels are decreased about 3 fold compared with WT mice, however CD8 expression levels were also normal in these mice (Legname et al., 2000). Analysis of the number of F5 CD8 T cells recovered, from the periphery of F5 Lck^{ind} ON and F5 WT mice showed that they were similar in the LN, but there was a substantial reduction in the number of CD8 T cells was

observed in the spleen (**Figure 3.2B**). In the F5 Lck^{ind} OFF group we observed a further reduction in the numbers of CD8 T cells both in the LN and spleen.

In summary the total number of F5 CD8 T cells in the periphery was significantly reduced in the partial and complete absence of Lck compared to F5 WT mice. It would seem that the different levels of Lck expression influences the total number of F5 CD8 T cells recovered from the periphery, and especially the proportion recovered from the spleen. Two important questions arise from this observation: 1) Is there a reduction in the export of mature T cells into the periphery in the partial and complete absence of Lck with a homing preference against the spleen? 2) Does Lck influence the survival of naïve CD8 T cells?

We observe an accumulation in the DN3 subset of the F5 Lck^{ind} ON mice, where some of the thymocytes may not be expressing the *lck* transgene (**Figure 3.1C**). In addition it has been shown the level of Lck expression in the DP thymocytes of polyclonal Lck^{ind} ON mice is heterogeneous, where some cells can express up to 10 fold increased levels of Lck compared with WT mice (Legname et al., 2000). In the inducible system, expression of the *lck* transgene restores the CD4 T cell repertoire comparable to WT levels however CD8 T cell numbers are reduced, altered Lck expression in the DP cells might be unfavourable for SP CD8 T cell selection (Legname et al., 2000). In our data we observe a 3 fold reduction in DP cells in the F5 Lck^{ind} ON mice and a 6 fold reduction in SP cells compared with the F5 WT mice (**Figure 3.1B**). There may be a link between the partial block in thymocyte development in the F5 Lck^{ind}

ON mice, and the reduced numbers of F5 CD8 T cells in the periphery compared with F5 WT mice.

In the F5 Lck^{ind} OFF mice, Lck expression was switched off only 7 days before analysis. Since the expression of Lck or Fyn have been shown to be required for naïve T cell survival in polyclonal mice (Seddon and Zamoyska, 2002b), we would expect the number of naïve CD8 T cells in the periphery to be the same as the F5 Lck^{ind} ON mice. The reduced number of F5 CD8 T cells observed in the complete absence of Lck expression points towards a stricter requirement for Lck signalling in naïve F5 CD8 T cell survival. A more detailed time course would need to be carried out in order to address this. The differential expression of Lck may also have an influence in the homing of F5 CD8 T cells preferentially to the LN over the spleen and this also requires further attention.

3.4 Lck expression influences CD5 expression in peripheral F5 CD8 T cells

We measured the cell surface expression of CD5 on F5 CD8 T cells from the LN and spleen and saw decreased expression of CD5 in the partial and complete absence of Lck compared with the F5 WT mice (**Figure 3.3**). CD5 expression has been shown to be influenced by Lck and Fyn in polyclonal mice and there was also a suggestion that there was a correlation of CD5 expression levels with cell survival (Seddon and Zamoyska, 2002b; Smith et al., 2001). Our data supports such a correlation with the CD5 expression and survival. We find total cell numbers in the periphery were reduced in the partial absence of

Lck (F5 Lck^{ind} ON) and further reduced in the complete absence of Lck (F5 Lck^{ind} OFF) expression (**Figure 3.2**) and CD5 expression was correspondingly decreased in the two populations (**Figure 3.3**).

Overall the analysis of the peripheral CD8 T cells in F5 mice showed that a relatively normal phenotype is generated in the partial and complete absence of Lck, despite the reduced cell recovery. CD8 expression levels were the same between the groups, although CD5 expression was lower in F5 Lck^{ind} ON and OFF cells. We then went on to analyse the response of peripheral naïve F5 CD8 T cells to antigen stimulation to test the requirement of Lck expression in the activation and generation of effector function.

3.5 Lck influences the expression of the early activation markers CD69 and CD25

Activation of T lymphocytes in response to a variety of stimuli *in vitro* and *in vivo* leads to the surface expression of CD69, a cell surface glycoprotein, which is upregulated as early as 2 hours post stimulation (Lopez-Cabrera et al., 1993). CD25 is also expressed on activated lymphocytes slightly later than CD69 (Waldmann, 1986). We addressed whether different levels of Lck affected the upregulation of these early activation markers. **Figure 3.4** shows that F5 Lck^{ind} ON and F5 Lck^{ind} OFF CD8 T cells displayed impaired upregulation of both CD69 (**Figure 3.4A**) and CD25 (**Figure 3.4B**) compared to F5 WT CD8 T cells. These results show that in the partial and complete absence of Lck, triggering of naïve F5 CD8 T cells upon stimulation with antigen is defective. In

agreement with previous studies the data suggests that the level of Lck expression determines the threshold for triggering cells into division (Lovatt et al., 2006; Seddon et al., 2000).

3.6 Lck influences the production of effector cytokines

Upon activation CD8 T cells differentiate and gain the capacity to produce cytokines. These include IL-2 an important autocrine cytokine produced and consumed by activated CD8 T cells which is required for cell survival and proliferation (Guidotti and Chisari, 2001). CTLs also produce cytokines TNF α and IFN γ , which have antiviral function (Guidotti and Chisari, 2001). We measured the production of these cytokines following F5 T cell activation to elucidate the contribution of Lck in their production. 24 hours post peptide stimulation TNF α production was measured in F5 CD8 T cells. Approximately 25% of F5 WT and F5 Lck^{ind} ON CD8 T cells produced TNF α , and in the absence of Lck a clear reduction in the proportion of cells (about 9%) able to make the cytokine was observed (**Figure 3.5A**). As the peptide concentration was reduced (**Figure 3.5B**), differences in the ability to make TNF α between the F5 WT and F5 Lck^{ind} ON groups also become apparent.

IL-2 production was measured at 24, 48 and 72 hours in the culture media of activated F5 CD8 T cells (**Figure 3.5C**). IL-2 detection from the F5 Lck^{ind} OFF CD8 T cells was markedly reduced compared to the F5 WT and F5 Lck^{ind} ON groups. In groups expressing Lck, IL-2 levels decreased over the 72 hour culture period, suggesting consumption of IL-2 exceeds production at these

later time points. In contrast IL-2 levels in the culture media of F5 Lck^{ind} OFF T cells never exceeded the baseline.

IFN γ production was measured following activation of F5 T cells with peptide for 72 hours *in vitro*. In the absence of Lck there was a marked reduction in both the percentage of CD8 T cells able to make IFN γ (**Figure 3.6 A & B**), and in the overall mean fluorescence intensity (MFI) (**Figure 3.6 C**).

The data shows cytokine production in general was not greatly impaired in activated F5 Lck^{ind} ON CD8 T cells, suggesting that levels of Lck expression does not affect the production of effector cytokines if the threshold for activation has been achieved, in contrast to the upregulation of CD69 and CD25, which appeared to be dependent on the level of Lck expression (**Figure 3.4**). At 24 and 48 hours following F5 CD8 T cell activation with low level and complete absence of Lck expression is not sufficient to fully upregulate the activation markers CD69 and CD25. However the signals received by F5 CD8 T cells expressing low levels of Lck is sufficient to programme the activated cells to produce similar levels of cytokines as the F5 WT CD8 T cells. Previously the level of Lck expression was shown to increase following activation if the culture media is supplemented with dox (Lovatt et al., 2006). Therefore the threshold for activating the production of IFN γ , which is observed 72 hours after stimulation, has been crossed.

It has been shown that in the absence of Lck CD4 T cells are unable to produce IL-2 upon stimulation, and that activation of the signalling molecule PLC γ located downstream of Lck and the ability to flux Ca²⁺ were severely impaired (Lovatt et al., 2006). In Jurkat cell lines the phosphorylation of PLC γ by PTKs upon TCR engagement is important for the activation of response elements found in the IL-2 promoter (Irvin et al., 2000). Our data confirm that for both CD8 and CD4 T cells the production of IL-2 driven through the TCR appears to be dependent on Lck expression.

In summary in the complete absence of Lck the number of cytokine producing F5 CD8 T cells was reduced compared to the F5 Lck^{ind} ON and F5 WT groups suggesting the threshold for activation was not achieved in these cells resulting in the reduced production of IL-2, TNF α and IFN γ *in vitro*.

3.7 Lck influences the development of CTL effector function

Since Lck activation and cytokine production was impaired in the absence of Lck, we then went onto assess the influence of Lck deficiency on effector generation *in vitro*. *In vitro* generated CTL were assayed for their ability to kill peptide pulsed target cells, from the EL4 thymoma cell line using the chromium release assay. First to determine the peptide dose required to generate optimal cytotoxic activity, WT F5 T cells were stimulated with different concentrations of NP68 for 72 hours. **Figure 3.7** shows that 0.1 μ M NP68 was a sufficient dose to generate maximal cytolytic activity. We decided to use 0.1 μ M NP68 to stimulate WT F5 T cells because there was no improvement in lytic ability at

higher peptide concentrations and more of the cells died. However, for the F5 Lck^{ind} ON and Lck^{ind} OFF CD8 T cells a higher dose of peptide, 1 μ M, was determined as the optimal concentration for each group (**Figure 3.4**) as they did not respond as efficiently to the lower dose. Following generation of CTL at optimal peptide concentrations, equivalent numbers of effector cells were cultured with the peptide pulsed target cells. F5 Lck^{ind} OFF CTLs showed a substantial decrease in their ability to kill target cells at the highest effector to target ratio (100:1) compared with F5 WT and F5 Lck^{ind} ON CTLs (**Figure 3.8A**).

The induction of target cell death can be divided into two common pathways Perforin/granzyme and Fas/FasL pathways (Lowin et al., 1994). The absence of Lck leads to a reduction in the development of cytotoxic function, which suggests that Lck may be important in inducing the expression of cytolytic proteins. Granule serine proteases known as granzymes are found within CTL and induce death in target cells in combination with perforin (Trapani and Smyth, 1993). We measured granzyme B production in the *in vitro* generated F5 CTL. F5 WT CTL expressed high levels of granzyme B (**Figure 3.8B**), with an MFI of 885. Lck^{ind} ON CTLs, also showed granzyme B expression comparable to F5 WT CTLs, with an MFI value of 952. In the absence of Lck, however, granzyme B production was reduced with fewer cells staining positive and the MFI of the CD8 T cell population was reduced to 273. The data shows in the complete absence of Lck expression the generation of functional CTL is impaired. In the presence of low level of Lck naïve F5 CD8 T cells require a higher dose of peptide to be optimally triggered compared with F5 WT CD8 T

cells. Although they have a reduced activation threshold F5 Lck^{ind} ON CD8 T cells are able to generate into fully functional CTL as assessed by the production of granzyme B, IFN γ and cytolytic activity.

3.8 Discussion

One of the first signalling molecules to be activated downstream of the TCR is Lck, a member of the Src family kinases (Straus and Weiss, 1992). How Lck influences the transmission of signals for generating effector and memory CD8 T cells is largely unknown. The aim of this study was to elucidate the role of Lck in the activation and generation of effector CD8 T cells using the Lck inducible model (Legname et al., 2000). Data presented in this chapter show that Lck is required for the activation and subsequent generation of effector F5 CD8 T cells *in vitro*.

The data show that Lck has different functions throughout the lifespan of a T cell from development (**Figure 3.1**) (Legname et al., 2000; Molina et al., 1992) to the differentiation of effector function of activated peripheral T cells (Legname et al., 2000; Lovatt et al., 2006; Seddon et al., 2000). In addition we show influence of F5 CD8 T cell survival maybe linked to CD5 levels (**Figure 3.2 & 3.3**).

The absence of proliferation compromises function, but it is not clear to what extent proliferation and differentiation are linked and whether both require identical signal transduction pathways. Studies have shown that the link

between the commitment to clonal expansion and effector cell differentiation is remarkably tight, for example the same duration of antigenic stimulation (2-24 hours) that drove naïve CD8 T cells to proliferate was sufficient for them to commit to differentiate into effector cells that could secrete IFN γ , TNF α and IL-2 and kill infected cells (Kaech and Ahmed, 2001; Mercado et al., 2000; van Stipdonk et al., 2003; van Stipdonk et al., 2001). Our data shows that the presence and level of Lck expression has a greater effect on CD25 and CD69, which are markers of triggering, suggesting the latter influences the threshold for activation (**Figure 3.4**) (Lovatt et al., 2006; Seddon et al., 2000). Upon achieving the required threshold for activation the generation of effector function was much less influenced by the level of Lck expression as demonstrated by the efficient production of effector cytokines and granzyme B and the subsequent killing of targets cell *in vitro* by F5 Lck^{ind} ON CTL (**Figure 3.8**).

We asked if F5 CD8 T cells in the complete absence of Lck were stimulated with a higher concentration of peptide would the threshold for triggering be enough to programme the cells into differentiation and acquisition of effector capabilities comparable to F5 WT levels. In the absence of Lck F5 CD8 T cells are difficult to stimulate, however some cells do overcome the activation threshold and some proliferation is observed, despite this they have poor effector function. Therefore inefficient signals through the TCR may lead to compromised activation of various transcription factors. For example it has been shown with polyclonal CD4 T cells that they are unable to induce IL-2

transcription in the absence of Lck (Lovatt et al., 2006), and it is therefore possible that NFAT activation is considerably reduced.

Similarly recent data has shown that naïve antigen inexperienced CD8 T cell precursors undergo marked changes in genetic remodelling which results in the expression of signature genes central to CD8 effector T cell function, including genes that encode cytokines and genes associated with cytotoxicity (Pearce et al., 2003). T-bet and eomesodermin (EOMES), members of the T-box family of transcription factors (Papaioannou, 1997; Pearce et al., 2003; Smith, 1997) have been shown to regulate the cytolytic effector mechanisms of CD8 T cells (Intlekofer et al., 2005; Pearce et al., 2003; Townsend et al., 2004). T-bet expression is rapidly induced in CD8 T cells by signalling through the TCR and the IFN γ R and it is required for the differentiation of antigen specific naïve CD8 T cells into effector CTLs, and also the production of IFN γ (Afkarian et al., 2002; Lighvani et al., 2001; Szabo et al., 2000). T-bet^{-/-} mice crossed with OT-1 TCR transgenic mice were shown to produce considerably less IFN γ upon antigen restimulation than WT mice (Sullivan et al., 2003). Interestingly these changes were masked if T cells were activated with PDBU and ionomycin, indicating that the strength of the signal transduced by the TCR has an important role. T-bet and EOMES have also been implicated in the regulation of perforin and granzyme B expression (Pearce et al., 2003; Sullivan et al., 2003). Retroviral overexpression of EOMES by T_H2 cells results in the induction of granzyme B expression, although it has not yet been shown that EOMES binds directly to the granzyme B promoter (Pearce et al., 2003). T-bet and EOMES have also

been shown to be responsible for CD122 expression, the receptor specifying IL-15 responsiveness, and mice deficient in both T-bet and EOMES were devoid of memory CD8 T cells (Intlekofer et al., 2005). Because T-bet expression is induced in CD8 T cells through the TCR and IFN γ R it is possible that in the absence of Lck expression inefficient signals through the TCR likely results in the inefficient expression of the T-bet and EOMES transcription factors. This would result in the phenotype we observed namely poor differentiation of activated CD8 T cells into armed effector cells, with reduced production of IFN γ , cytotoxic proteins such as granzyme B and ultimately defective target cell killing (**Figure 3.6 and 3.8**). Therefore we propose in order to meet the threshold for activation of naïve CD8 T cells, Lck expression is the limiting factor. If the CD8 T cell is sufficiently activated the cell will undergo clonal expansion and differentiation into effector CD8 T cells. Subsequently leading to the efficient transcription of genes involved in cytotoxicity via the transcription factors such as NFAT, T-bet and EOMES.

The issue of incomplete differentiation of CTL precursors in response to subdominant viral antigens (Spencer and Braciale, 2000) or those involving low affinity anti-tumour CD8 T cells (Dutoit et al., 2001) have been addressed. In addition the strength of TCR engagement (Gett et al., 2003) and the cytokine milieu (Manjunath et al., 2001) together have been shown to influence the differentiation of naïve CD8 T cells upon activation. More recently a study showed poor functional effector CD8 T cells were generated in response to weak antigen stimulation (Verdeil et al., 2006). Here Verdeil et al demonstrated

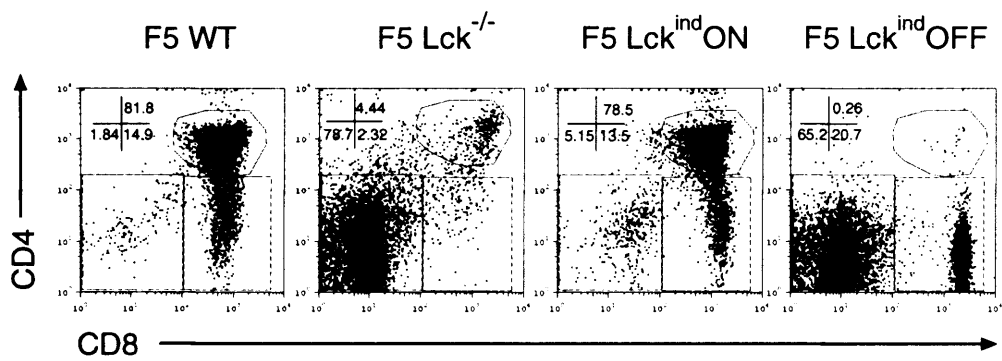
transient gene expression for a cluster of genes including CD25, granzyme B and TNF-R family costimulatory molecules (GITR, OX40 and 4-1BB) in response to weak TCR agonists. Gene expression was however stabilised by the addition of IL-2. The STAT5 transcription factor is important in modulating T cell functions through the IL-2R (Ihle, 2001). Also TCR stimulation dependent on Lck expression has been shown to directly activate STAT5 (Welte et al., 1999). In the study by Verdeil et al a constitutively active form of STAT5 mimicked the effect of IL-2 by sustaining transcripts for the same gene cluster under conditions of low TCR avidity and IL-2 treatment. Endogenous STAT5 binding to 4-1BB and granzyme B promoters was shown and so IL-2 via the activation of STAT5 acts as a stabiliser of gene regulation for these genes, initiated by TCR signals thus contributing to the development of a complete CD8 T cell effector program. It is possible F5 Lck^{ind} OFF CD8 T cells upon antigen activation appear to also mimic the effect of weak TCR stimulation by the poor production of IL-2 resulting also in the inefficient activation of STAT5. This in turn could lead to the incomplete differentiation of CD8 T cells and impaired cytolytic function. It would be interesting to see if the addition of exogenous IL-2 to the cultures could restore the proliferation and hence differentiation of F5 CTLs generated in the absence of Lck, to improve their effector function. In the future, it would also be interesting to carry out experiments to determine if the activation and expression of transcription factors involved in mediating effector functions of CTL are affected in the partial or complete absence of Lck expression.

Figure 3.1: Thymocyte development is restored in F5 Lck deficient mice upon introduction of an *lck* transgene

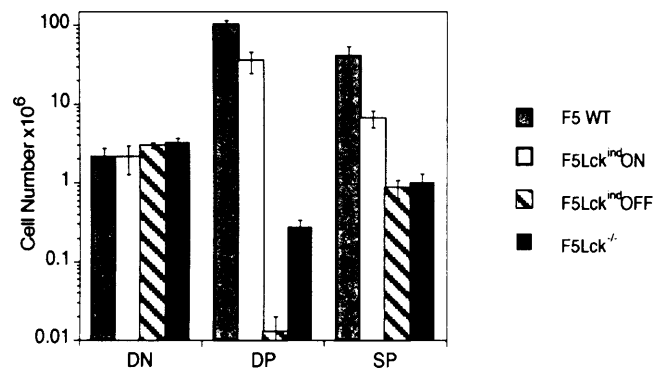
Thymocytes were analysed from 5 week old F5 TCR transgenic mice on a Rag1^{-/-} background (F5 WT), a Rag1^{-/-} Lck^{-/-} (F5 Lck^{-/-}) background or expressing an inducible lck transgene on a Rag1^{-/-} Lck^{-/-} background (F5 Lck^{ind}), which were either continuously fed dox (F5 Lck^{ind} ON) or were taken off dox at least one week before (F5 Lck^{ind} OFF). F5 Lck^{-/-} Rag1^{-/-} mice were 10 weeks old.

(A) The percentage of total cells in each sub-population are shown in top left had side of the FACS plots. Thymocytes were stained with antibodies to CD4 and CD8. (B) The total cell numbers of the DN, DP and mature CD8 SP populations are presented. (C) The frequency of DN cells (gated on CD4 and CD8 negative) were further analysed based on their CD44 and CD25 profiles. (D) The absolute cell numbers for the different DN subsets were calculated. A minimum of 100,000 total events was collected per sample. Each graph shows the mean absolute cell number (n=3) ± SD. This data is representative of 3 independent experiments.

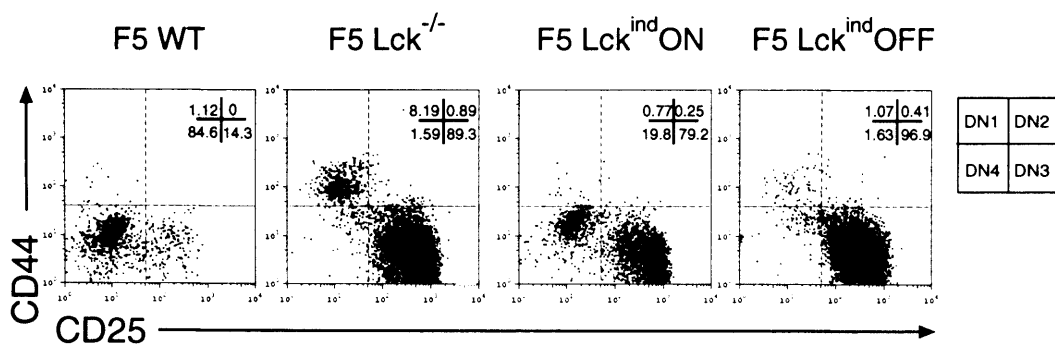
A



B



C



D

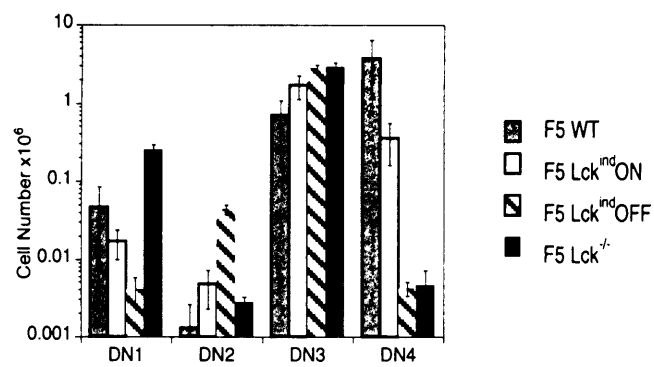
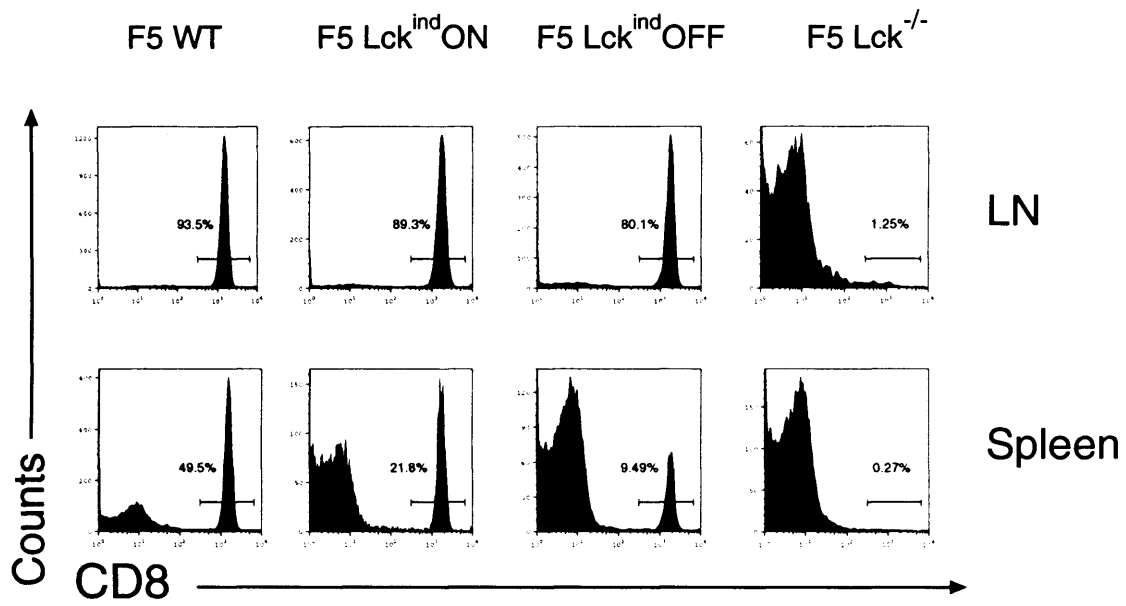


Figure 3.2: *lck* transgene expression restores development of peripheral F5 *Lck*^{-/-} T cells

Cell suspensions from LN and spleen of F5 WT, F5 *Lck*^{ind} ON, F5 *Lck*^{ind} OFF and F5 *Lck*^{-/-} mice were stained with antibodies to CD8 and the frequency of CD8 T cells was measured (A). Absolute CD8 T cell numbers were calculated and shown in (B). Cells were analysed by flow cytometry and a minimum of 20,000 total events were collected. The graph shows the mean cell numbers calculated (n=3) ±SD. Data is representative of 3 independent experiments. Statistical significance was calculated based on total cell number using the Mann Whitney test (P< 0.05 = * P< 0.01 = **)

A



B

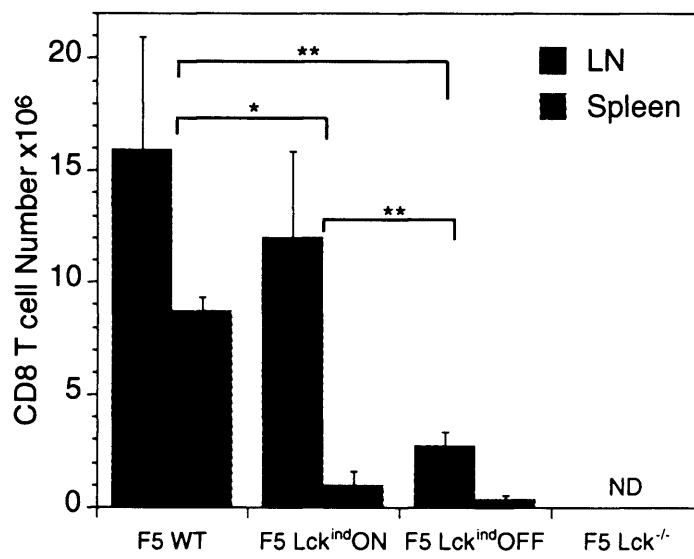
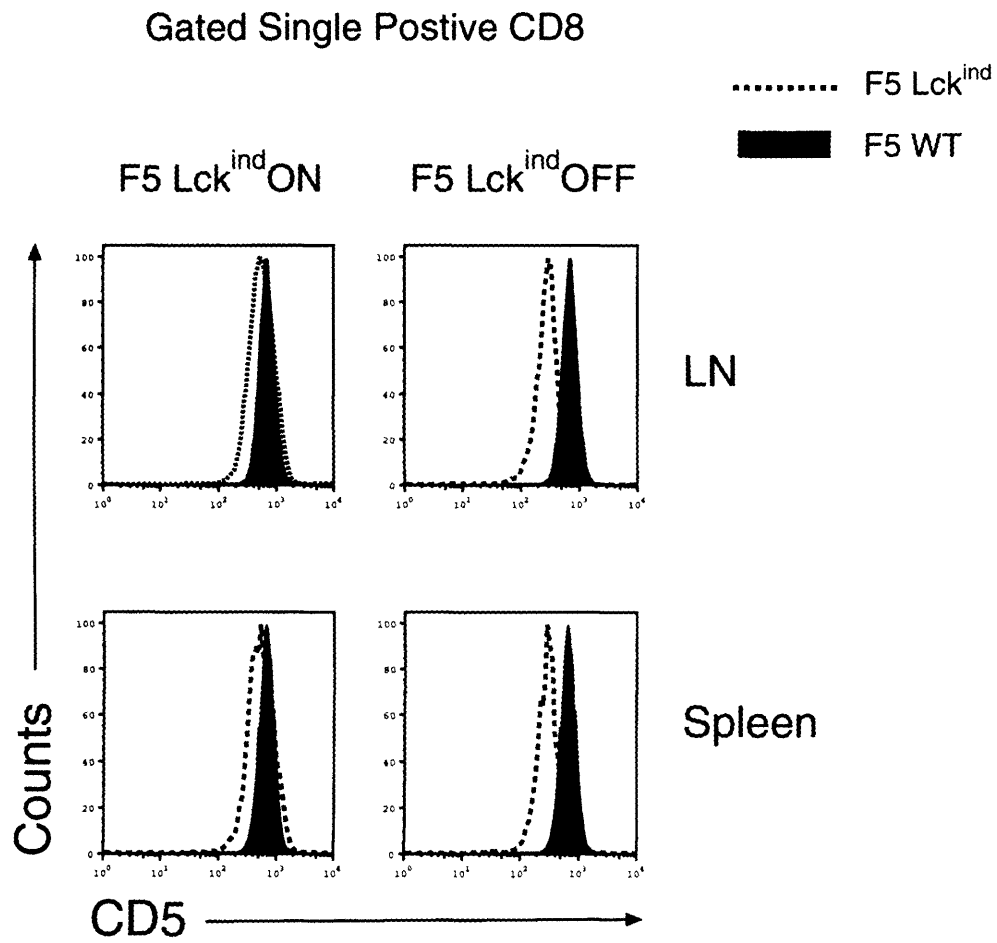


Figure 3.3: CD5 expression is lower in F5 Lck^{ind} T cells

Peripheral lymphoid cells were evaluated for CD5 expression in F5 Lck^{ind} ON and OFF mice were compared with F5 WT mice. (A) CD5 expression on gated single positive CD8 T cells is shown. (B) The average CD5 MFI \pm SD is shown graphically. The cells were analysed by flow cytometry and a minimum of 20,000 events were collected. The data is representative of 3 independent experiments.

A



B

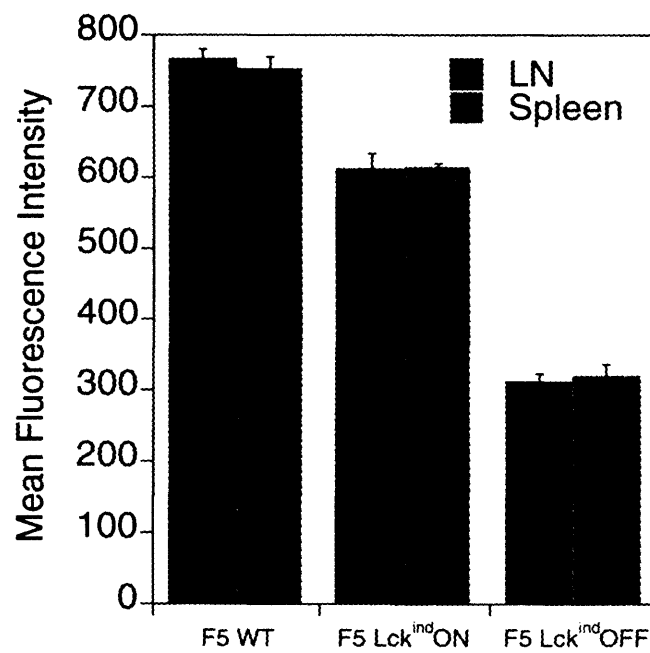
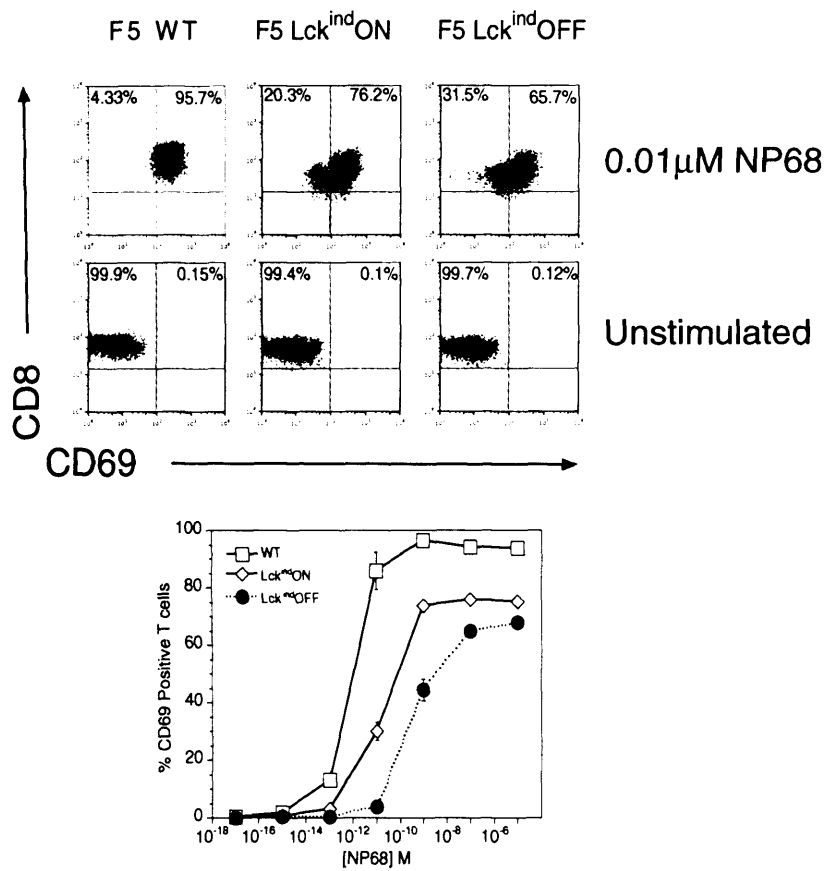


Figure 3.4: Reduced upregulation of CD69 and CD25 in the absence of Lck

LN derived F5 CD8 T cells in the presence or absence of Lck were stimulated with a titration of NP68 peptide antigen *in vitro*. (A) Cells were stimulated for 24 hours to measure CD69 and (B) 48 hours to measure CD25 expression. The percentage of gated F5 CD8 T cells that were positive for CD69 and CD25 is shown as FACS plots for one peptide concentration 0.01 μ M, together with the unstimulated control. The graphs show mean (n=3) \pm SD gated F5 CD8 T cells positive for (A) CD69 and (B) CD25 in response to peptide. The gates were applied based on unstimulated controls. The data is representative of 5 independent experiments.

A



B

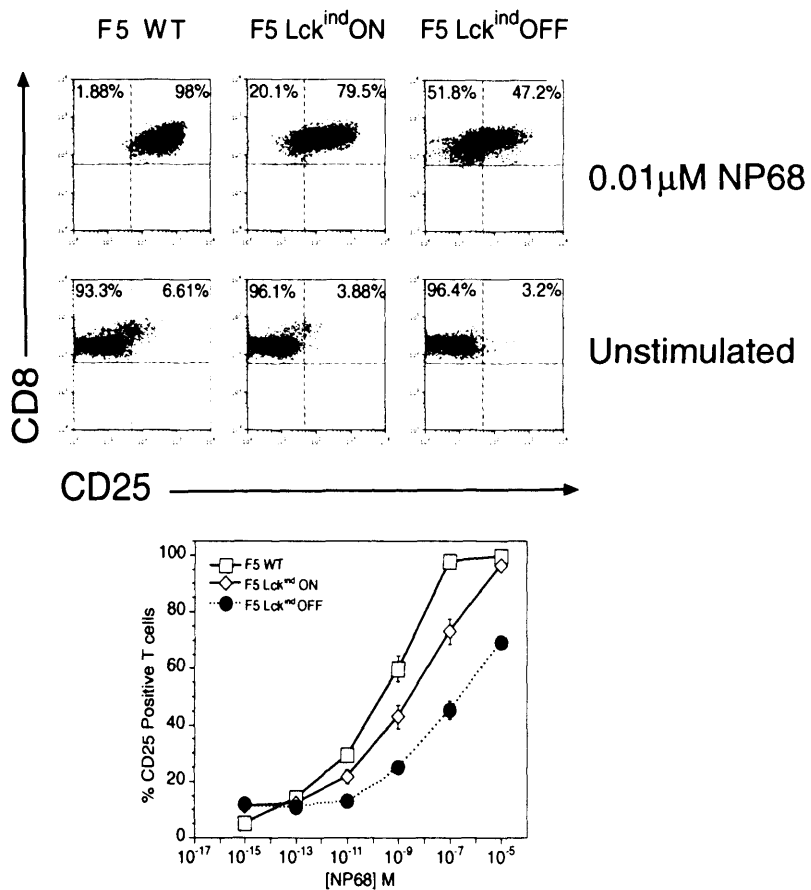
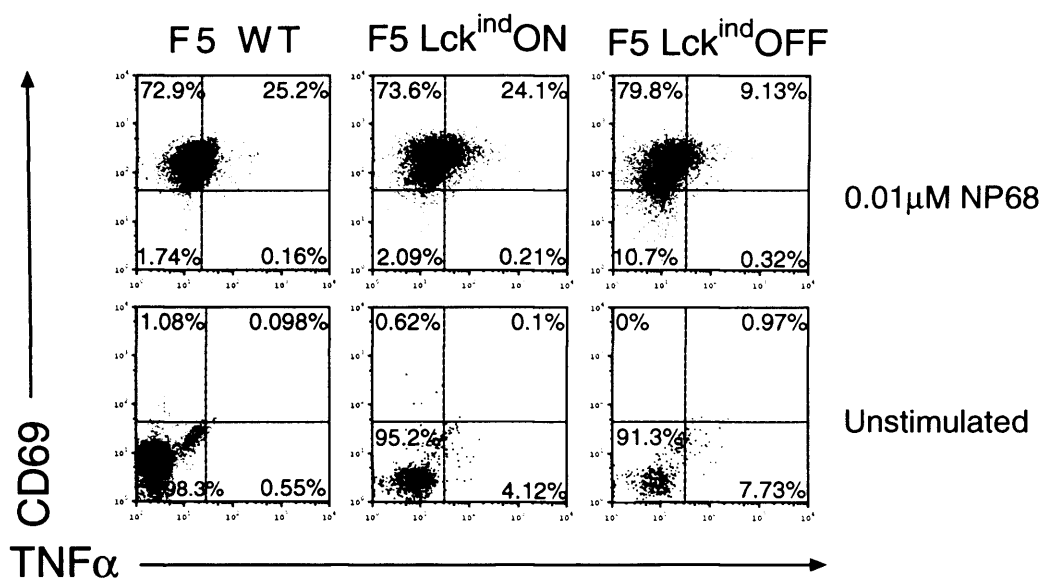


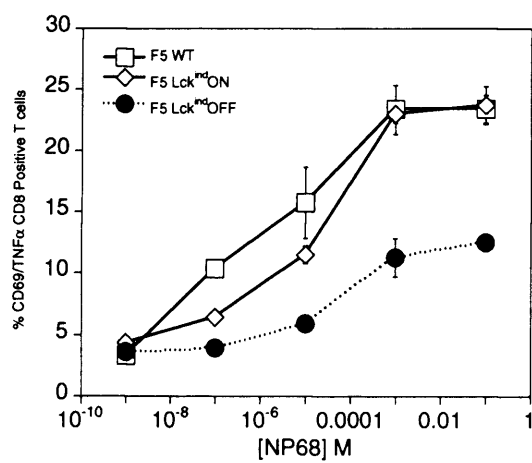
Figure 3.5: Lck expression influences TNF α and IL-2 production

(A) TNF α production was measured by intracellular staining of activated F5 T cells at 24 hours and analysed by flow cytometry. Lymph node cells were stimulated with peptide for 24 hours to measure TNF α production. F5 cells stimulated with 0.01 μ M NP68 are shown in the upper panel of FACS plots with un-stimulated controls in the lower panel. (B) The graph shows the mean (n=3) \pm SD of gated CD69/TNF α positive F5 CD8 T cells in response to peptide. The gates were set on un-stimulated controls as shown in (A). Data is representative of 5 independent experiments. (C) IL-2 secretion was measured from the supernatants of F5 T cells activated with 0.01 μ M peptide at 24, 48 and 72 hour time points. Graph shows the mean production of IL-2 units/ml (n=3) \pm SD calculated from a standard curve. Data is representative of 5 independent experiments.

A



B



C

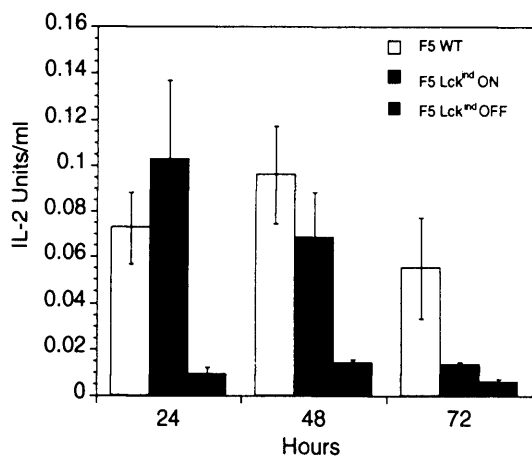
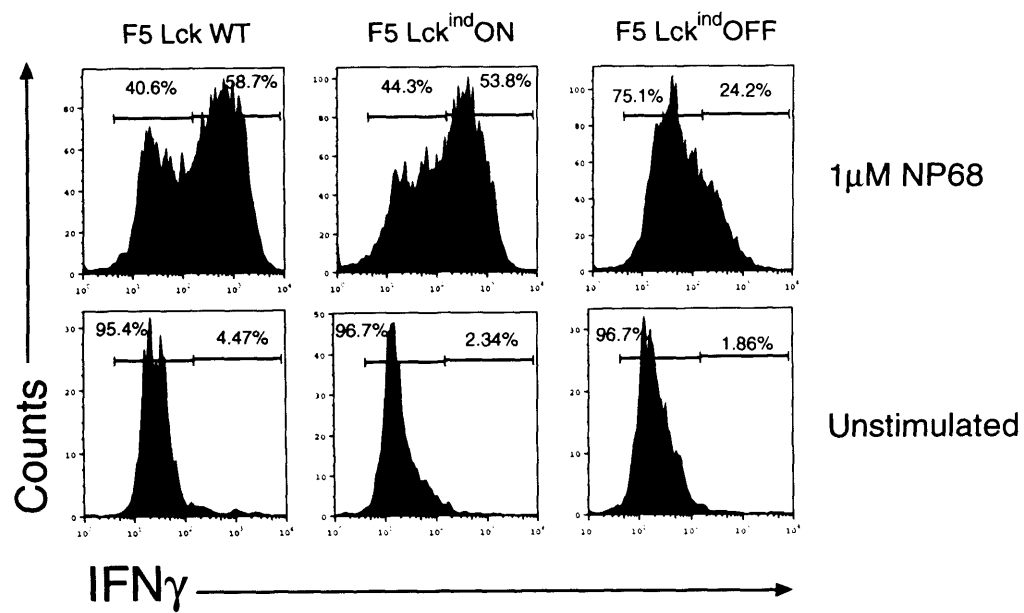


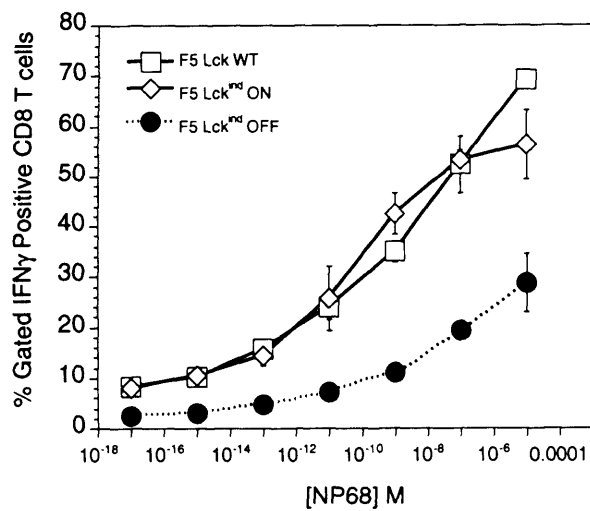
Figure 3.6: IFN γ production is reduced in the absence of Lck

(A) IFN γ production was measured by intracellular staining of activated F5 T cells at 72 hours and analysed by flow cytometry. Lymph node cells were simulated with peptide for 72 hours to measure IFN γ production, and cells stimulated with 1 μ M NP68 are shown in the upper panel and unstimulated controls in the lower panel. The graph shows the mean (n=3) \pm SD of gated IFN γ positive F5 CD8 T cells in response to peptide. The gates were set on unstimulated controls. (B) Shows the total mean fluorescence intensity for IFN γ production on gated IFN γ positive F5 CD8 T cells. The data is representative of 3 independent experiments.

A



B



C

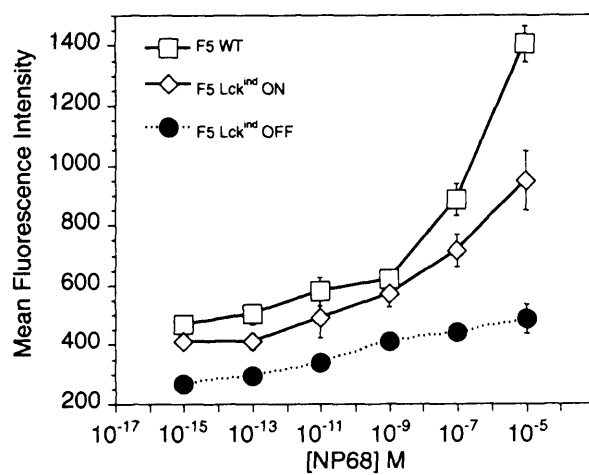


Figure 3.7: Functional CTL are generated after 72 hours stimulation with NP68 peptide antigen in F5 WT mice

F5 WT were stimulated *in vitro* for 72 hours with a titration of NP68 peptide to determine the concentration for optimal CTL. CTL were cultured with EL4 target cells loaded with NP68 peptide antigen and labelled with Na⁵¹Cr for 4 hours. The graph shows specific lysis of target cells at varying effector:target ratios from the mean of triplicate wells \pm SD. Data is representative of 3 independent experiments.

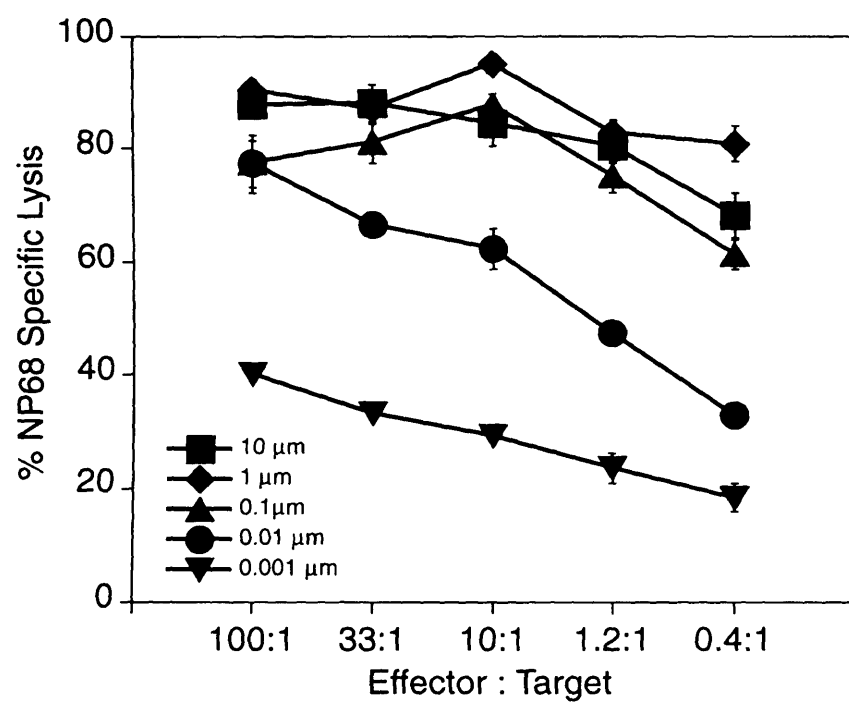
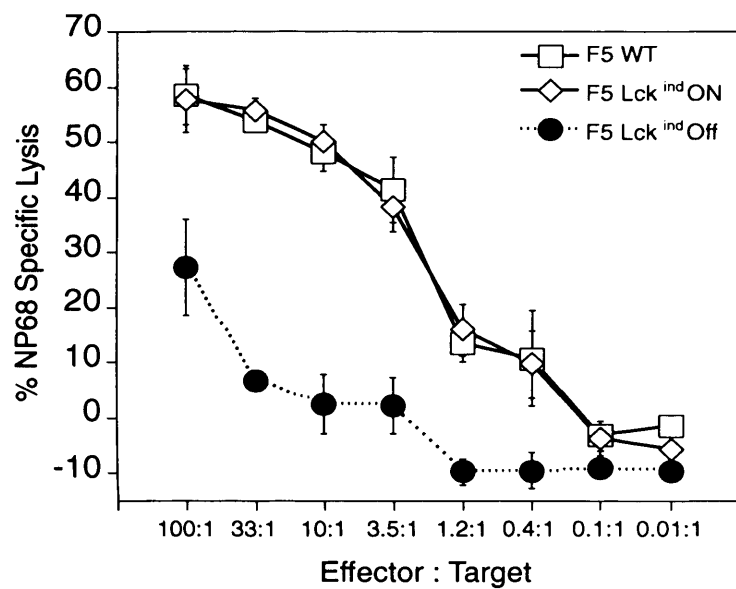


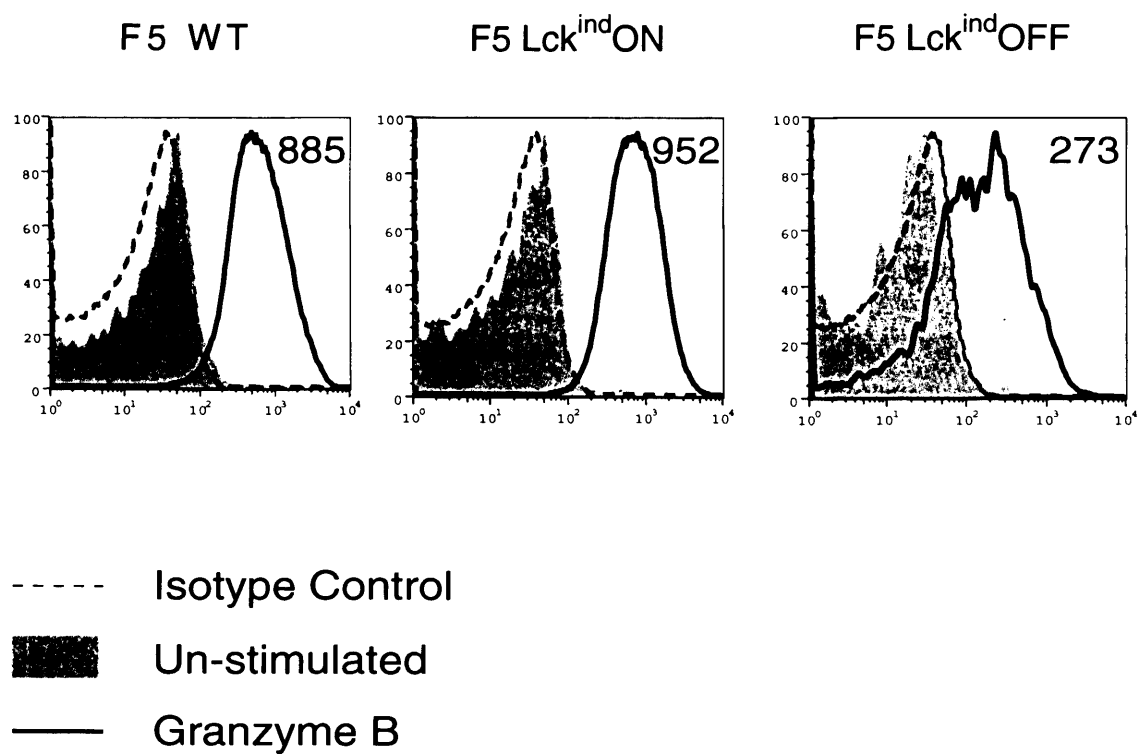
Figure 3.8: Poor generation of CTL and defective production of Granzyme B in the absence of Lck

CTL were generated from splenic F5 T cells. F5 WT cells were stimulated with 0.1 μ M NP68 and F5 Lck^{ind} T cells with and without Lck were stimulated with 1 μ M NP68 for 72 hours and incubated with peptide and Na⁵¹Cr loaded EL4 targets cells labelled with for 4 hours. (A) Graph shows percentage specific lysis of target cells for triplicate wells \pm SD. The data is representative of 3 independent experiments. (B) Histograms show the relative expression of Granzyme B. MFI values for granzyme B, calculated for total CD8 T cells are shown in the top right hand corner of each histogram.

A



B



Chapter 4: The role of Lck in the generation of effector CD8 T cells *in vivo*

4.1 Introduction

Successful activation of naïve CD8 T cells by antigen presented by MHC, leads to a programme of clonal expansion and the differentiation of effector function into armed CTLs. *In vitro* studies using F5 CD8 T cells (**Chapter 3**) and experiments conducted with polyclonal CD4 T cells have shown that upon stimulation in the partial or complete absence of Lck, naïve T cells were not triggered efficiently, demonstrated by the reduced expression of the early activation markers CD69 and CD25 (**Figure 3.4**) (Lovatt et al., 2006; Seddon et al., 2000). We hypothesise that as a consequence F5 CD8 T cells were unable to differentiate into functional CTL, as confirmed by reduced cytolytic function and production of granzyme B (**Figure 3.8**).

We extended these studies to an *in vivo* system in which mice were challenged with live A/NT/60-68 influenza virus (Moskophidis and Kioussis, 1998) to further elucidate the requirement of signalling through Lck upon activation of naïve F5 CD8 T cells in a physiological environment. Studies have been carried out previously to address the role of Lck signalling in the generation of effector CD8 T cells. Early work by Molina et al challenged polyclonal Lck^{-/-} mice with recombinant vaccinia and LCM virus, and showed that the small number of CD8 T cells which were present in the periphery of these mice were unable to protect

the mice from infection (Molina et al., 1993). However these experiments did not fully address the requirement for Lck in CD8 T cell effector function since it is possible that the profound reduction of T cell number in the periphery prevented clearance of the virus. Trobridge et al developed an Lck transgenic mouse, which expressed functional Lck in the thymus but not in mature peripheral T cells (Trobridge and Levin, 2001). CTL were generated from these Lck mutant mice by stimulating splenocytes with allogeneic BALB/c splenocytes and were tested for their ability to lyse ^{51}Cr -labeled target cells of the H-2^d haplotype. The CTL from Lck mutant mice lysed target cells to a similar extent as those generated from control T cells. More recently Tewari et al addressed the role of Lck in the *in vivo* generation of anti-viral effector CD8 T cells using the polyclonal Lck^{ind} system developed in our laboratory, they showed the requirement for Lck-mediated TCR signalling in antigen specific CD8 T cell responses during acute viral infections. Lck deficient CD8 T cells failed to activate and clonally expand upon challenge with virus (Tewari et al., 2006). However, this study did not address the primary effector function of CTL with respect to the generation of IFN γ production or cytolytic ability.

To examine the role of Lck in the generation of CD8 T cell effector function *in vivo*, we established an adoptive transfer system, where a fixed number of naïve F5 CD8 T cells were co-transferred into Rag1^{-/-} host mice with live influenza A/NT/60-68 virus. This allowed us to precisely measure the expansion of a fixed number of antigen-specific cells in mice expressing different levels of Lck. We tried to activate the F5 CD8 T cells with peptide

pulsed DCs (data not shown), however the cells were not activated efficiently. Therefore the live influenza A/NT/60-68 virus was used and was shown to work well. In our experiments 4 parameters were measured: activation, expansion, differentiation and mediation of effector function. Here we show in agreement with previous studies that Lck signalling is required for the activation and expansion of F5 CD8 T cells *in vivo* (Tewari et al., 2006). However we have also demonstrated that in the complete absence of Lck expression F5 cells were very defective in their ability to elicit cytolytic function and showed reduced production of IFN γ upon re-stimulation *in vitro*.

4.2: Response of F5 WT CD8 T cells following immunisation with influenza virus

To study the role of Lck in the generation of effector CD8 T cells *in vivo* we activated F5 WT CD8 T cells with the live influenza virus A/NT/60-68. F5 WT CD8 T cells together with different doses of influenza virus were adoptively transferred into Rag1^{-/-} host mice. **Figure 4.1A** shows that all F5 WT CD8 T cells were activated with 20HA Units/mouse, and all homogenously up-regulated CD44. A fuller titration of the flu virus was also carried out and was expressed as cell recovery (**Figure 4.1B**). With 20HA units of the virus all F5 WT CD8 T cells were activated, with optimal recovery, however at lower doses of the virus not all the cells were optimally activated as demonstrated by reduced cell recoveries and failure to fully upregulate CD44. At higher doses of virus > 20HA units all the cells were activated but the recovery was reduced suggesting that 100 HA units of the virus was an excessive dose, and resulted

in cell death (**Figure 4.1B**). We therefore decided to challenge all host mice with a dose of 20HA units of the virus to study the role of Lck in the activation and expansion of transferred F5 CD8 T cells *in vivo*.

4.3: Triggering of F5 CD8 T cells is dependent on the presence and amount of Lck expression

The data presented in Chapter 3 demonstrated that Lck is required for the activation and generation of effector function *in vitro*. In addition, data from Tewari et al suggested that in the absence of Lck polyclonal CD8 T cells failed to activate and expand as there was reduced cell recovery upon viral challenge compared with Lck sufficient CD8 T cells (Tewari et al., 2006). We addressed whether the same was true for F5 CD8 T cells challenged with flu virus by measuring their proliferation *in vivo*. CFSE labelled F5 CD8 T cells were transferred into Rag1^{-/-} host mice together with influenza virus and cell recoveries and CFSE dilution were monitored at day 1, 3 and 8 post transfer. F5 CD8 T cell recoveries from each group at day 1 was equivalent irrespective of Lck expression, demonstrating that the process of adoptive transfer did not affect the degree of engraftment between the different groups (**Figure 4.2A**). Three days following transfer proliferation was detected in response to antigen in the F5 WT and F5 Lck^{ind} ON CD8 T cells (**Figure 4.2B**). The F5 Lck^{ind} ON CD8 T cells proliferated to a lesser extent than the F5 WT CD8 T cells, while the F5 Lck^{ind} OFF CD8 T cells failed to proliferate at this time point (**Figure 4.2B & D**). The percentage of F5 CD8 T cells recovered from each group was similar, however when converted to absolute numbers fewer cells were

recovered from F5 Lck^{ind} ON mice and fewer still in the complete absence of Lck compared to the F5 WT control (data not shown). **Figure 4.2C** shows the percentage of F5 CD8 T cells, which had undergone division, was 6 fold higher in F5 WT CD8 T cells compared to F5 Lck^{ind} OFF CD8 T cells and 2 fold higher compared to the F5 Lck^{ind} ON CD8 T cells. Together the data show a requirement for Lck in the induction of proliferation of F5 CD8 T cells. It is possible that in the absence of Lck the threshold for triggering cells into division is raised, and the presence of high dose of virus is unable to overcome this threshold.

By day 8 most F5 WT and F5 Lck^{ind} ON CD8 T cells had initiated proliferation, and all T cells had successfully upregulated CD44 expression (**Figure 4.3A**). However in the absence of Lck a substantial proportion of the F5 CD8 T cells remained undivided, and had not fully upregulated CD44 expression. Therefore Lck deficient T cells are able to proliferate but take longer to do so. As a consequence the CD8 T cell recoveries by day 8 were significantly reduced when Lck expression was low or completely absent compared with the F5 WT group (**Figure 4.3B**). This experiment has been carried out twice and needs to be repeated to confidently determine the degree of expansion is dependent on the level of Lck expression.

4.4: Lck is required for the activation and expansion of F5 CD8 T cells

Activated F5 CD8 T cells were recovered from the LN and spleen at day 8, from each of the groups described above. In addition a further control group was set

up in which F5 WT CD8 T cells were transferred into Rag1^{-/-} recipient mice in the absence of virus so that the influence of a lymphopenic environment could be assessed. **Figure 4.4A** shows the FACS plots for F5 CD8 T cell recoveries for each group at day 8. The proportion of F5 CD8 T cells recovered in both the LN and spleen was highest in F5 WT mice, was significantly reduced in the F5 Lck^{ind} ON mice, and was further reduced in F5 Lck^{ind} OFF mice. The cell recovery from the group in which unimmunised F5 WT CD8 T cells were transferred, was similar to F5 Lck^{ind} OFF group (**Figure 4.4B**).

We measured the cell surface expression of CD44 to investigate the activation of F5 CD8 T cells. All F5 WT and F5 Lck^{ind} ON CD8 T cells upregulated CD44 (**Figure 4.5A**); however, almost 30% of F5 Lck^{ind} OFF CD8 T cells failed to upregulate CD44, suggesting that not all of the CD8 T cells in the absence of Lck had been primed. These results are in contrast to experiments conducted by Tewari et al who show very few activated CD8 T cells in the absence of Lck in their system (Tewari et al., 2006). In addition their data does not clearly show whether the few Lck^{ind} OFF CD8 T cells recovered were able to upregulate CD44 (Tewari et al., 2006) and this observation is probably a limitation of using a polyclonal system in which the starting frequency of antigen specific T cells is low. We also found that a small proportion of F5 WT CD8 T cells transferred with no flu, had upregulated CD44. This observation was consistent with studies showing that the transfer of naïve T cells into lymphopenic host mice results in proliferation and a partially activated phenotype induced by the lymphopenic environment (Cho et al., 2000; Ernst et

al., 1999; Goldrath and Bevan, 1999). Lymphopenia-induced proliferation is mediated by signals through the TCR, which are dependent on the expression of Lck (Seddon et al., 2000). Transferring unimmunised F5 Lck^{ind} OFF into lymphopenic host mice did not induce proliferation (Seddon and Zamoyska, 2002a) nor the upregulation of CD44 (unpublished data). We therefore concluded that upregulation of CD44 in F5 Lck^{ind} OFF CD8 T cells was induced by antigen and not as a consequence of being in a lymphopenic environment. Despite the similar cell recoveries the number of F5 Lck^{ind} OFF CD8 T cells, which had upregulated CD44, was higher in comparison to the F5 WT unimmunised group (**Figure 4.5**). Furthermore a greater proportion of F5 CD8 T cells recovered from the spleen upregulated CD44 expression in the F5 Lck^{ind} OFF and F5 WT unimmunised groups compared with those recovered in the LN (**Figure 4.5B**).

4.5: Defective expansion of activated F5 CD8 T cells in the absence of Lck in full hosts

In order to study an easily identifiable fixed cohort of cells our studies were mainly carried out using lymphopenic host mice. In the absence of Lck F5 CD8 T cells do not expand in a lymphopenic environment (Seddon and Zamoyska, 2002a), however it was clear that F5 WT unimmunised cells did expand and developed some effector function (described later). Therefore it was important to distinguish the influence of lymphopenia-induced proliferation from that induced by antigen. In addition we wanted to establish whether the presence of host cytokines and other cell types such as CD4 T cells would help the

response of F5 CD8 T cells to antigen. Allotype marked F5 WT CD45.1/2 and F5 Lck^{ind} (CD45.2) CD8 T cells were mixed in equal ratios and transferred into intact tolerant host mice together with the influenza A/NT/60-68 virus. After 7 days the host mice were sacrificed and the transferred F5 CD8 T cells were analysed for activation marker expression and cell recovery (**Figure 4.6**).

F5 WT CD45.1/2 CD8 T cells expanded to virus in the intact mice and the F5 Lck^{ind} ON CD8 T cells also expanded as before but were less efficient (**Figure 4.6A & B**). F5 Lck^{ind} OFF CD8 T cells did not expand upon activation with the influenza virus and there was no expansion of transferred F5 CD8 T cells in mice that were unimmunised (**Figure 4.6B**). We also measured cell surface expression of CD44 upon activation, F5 WT and F5 Lck^{ind} ON CD8 T cells showed similar levels of surface CD44 upregulation. In the F5 Lck^{ind} OFF CD8 T cells however, CD44 up-regulation was impaired, and very few cells were recovered (**Figure 4.6B & C**). These observations were in agreement with studies conducted in the lymphopenic host mice. The data confirms that despite the lymphopenic host environment the response to virus is comparable to that observed in an intact animal. The host environment does not change the results we have observed showing that in the absence of Lck expression F5 CD8 T cells are inefficiently activated and undergo reduced expansion compared to the Lck sufficient F5 CD8 T cells. As before the degree to which the F5 CD8 T cells expand is also dependent on their level of Lck expression.

4.6: Reduced killing of target cells in the absence of Lck

In order to measure the ability of antigen stimulated F5 CD8 T cells to kill target cells, LN cells from C57BL/6 CD45.1 mice were transferred into the host mice at day 6 as illustrated in **Figure 4.7A**. The targets cells were pulsed with different concentrations of NP68: 2.5×10^{-6} M (Hi), 2.5×10^{-9} M (Med), 2.5×10^{-12} M (Lo) or no antigen (0) and were labelled with different concentrations of CFSE so that they could be distinguished from each other. The target cells were mixed in equal ratios and were transferred into the experimental host mice. Target cells were also transferred into naïve F5 Rag1^{-/-} mice as controls, this was to ensure that lysis of target cells by F5 CTL was specific. These cells were recovered in their starting proportions from F5 Rag1^{-/-} mice, and these recoveries were used as the baseline control from which to calculate specific killing (**see methods and materials section 2.9.3**). The data show equivalent recovery of the presence of the no peptide peak from all groups, confirming that lysis of target cells was antigen specific (**Figure 4.7B**). At high peptide concentrations similar killing of target cells was observed between F5 WT and F5 Lck^{ind} ON CTL (**Figure 4.7B & C**). However F5 Lck^{ind} ON CTL were slightly less efficient at killing target cells pulsed with an intermediate dose of peptide. In this experiment F5 Lck^{ind} OFF CTL were unable to mediate target cell lysis, similarly F5 WT T cells, which were transferred in the absence of influenza virus showed only background cytolytic ability (**Figure 4.7B & C**). However, in some experiments low level of target cell lysis above background was observed (data not shown). At the very lowest peptide pulsed concentrations, none of the F5 CTLs were able to kill the target cells. One explanation for these results is that

target cells need to present a specific amount of peptide in order for efficient recognition and activation of CTL. Our data indicates in agreement with the *in vitro* experiments that in the absence of Lck F5 CTL were unable to mediate effective killing of target cells (**Figure 3.8A**). Therefore F5 CD8 T cells need to be able to efficiently signal through the TCR in order to generate into fully function CTL to mediate cell lysis. This has been addressed further in **Chapter 5**.

4.7: The production of IFN γ is reduced in the absence of Lck

The ability of CTL to make IFN γ is an important effector function for the successful clearance of viral infection (Schroder et al., 2004). We asked whether in the absence of Lck expression, IFN γ production was impaired or different from CTL activity (**Figure 4.8**). The percentage of CD8 T cells able to produce IFN γ was similar for F5 WT and F5 Lck^{ind} ON CD8 T cells, but was reduced in the F5 Lck^{ind} OFF and in the F5 WT unimmunised group (**Figure 4.8A and B**). The observations that the unimmunised group are able to produce IFN γ is consistent with reports of naïve T cells that are introduced into a lymphopenic environment they can differentiate and gain effector function such as the ability to make IFN γ (Cho et al., 2000; Goldrath et al., 2004; Hamilton et al., 2006). When we measured the MFI values for IFN γ , the F5 Lck^{ind} ON, F5 Lck^{ind} OFF and F5 WT unimmunised CD8 T cells produced comparable amounts of IFN γ and was only slightly increased in comparison with the naïve F5 Rag1^{-/-} control (**Figure 4.8C**). F5 WT CTL produced a higher amount of IFN γ compared to the other groups (**Figure 4.8C**). The data shows

that the requirement for Lck expression is less stringent for low level of IFN γ production, however to produce a higher amount of IFN γ , effector F5 CD8 T cells need to express WT levels of Lck.

4.8: Discussion

The aim of this study was to elucidate the role of Lck in the priming, expansion and generation of effector function of F5 CD8 T cells *in vivo*. Here we have shown that the activation and expansion of F5 CD8 T cells is dependent on the expression of Lck, and the level of expression appears to determine the magnitude of expansion. In addition we have found that in order to mediate effector function Lck expression is also required but this varies depending on the parameters assessed.

Studies have shown that various parameters can set the threshold for triggering naïve T cells into proliferation and differentiation. For example, the concentration and affinity for antigen (Valitutti et al., 1995; Viola and Lanzavecchia, 1996), the presence or absence of co-stimulation, and the duration of the interactions of T cells with DCs, together can determine the outcome of the signalling process (Iezzi et al., 1998; Iezzi et al., 1999). We have shown *in vitro* that the partial and complete absence of Lck expression also raises the threshold for triggering polyclonal naïve T cells (Lovatt et al., 2006; Seddon et al., 2000) and F5 CD8 T cells (**Chapter 3**). In this chapter we have extended our *in vitro* observations into an *in vivo* system. A fixed number of F5 CD8 T cells were challenged with influenza virus, and were subsequently

monitored for their ability to activate, expand and differentiate into CTL *in vivo*. We demonstrate that F5 Lck^{ind} OFF CD8 T cells require more time in the presence of antigen before being triggered into proliferation (**Figure 4.2**) and this in turn reflects the magnitude of expansion (**Figure 4.3 and 4.4**).

Our data agree with work by Tewari et al who have reported that limiting Lck expression at defined time points during the priming phase determined the magnitude of the primary response (Tewari et al., 2006). Prlic et al also showed that the duration of the initial TCR stimulus controlled the magnitude but not the effector function of the CD8 T cell response (Prlic et al., 2006). Our work indicates that there are different thresholds to achieve particular effector functions and Lck influences which thresholds are reached (Lovatt et al., 2006; Seddon et al., 2000), but it also appears that different thresholds are set for mediating effector functions of F5 CTL. We have shown the ability to produce IFN γ is less dependent on the expression of Lck than the ability to elicit target cell killing as similar numbers of cells were capable of making IFN γ . However there was also an influence of Lck in the amount of IFN γ produced per cell. Hence, a lower threshold for triggering F5 CTL in the partial and complete absence of Lck maybe required to produce a small amount of IFN γ upon restimulation, whereas WT expression of Lck expression results in a high production of IFN γ . IFN γ production from *in vivo* generated F5 CTL was different from the results from the *in vitro* activated F5 CD8 T cells where F5 Lck^{ind} ON CD8 T cells produced similar levels of IFN γ to the F5 WT CD8 T cells.

It is possible this difference is due to the level and duration of antigen stimulation, which may be more localised and sustained *in vitro*.

With respect to the ability to trigger the lytic machinery, F5 WT CTL and F5 Lck^{ind} ON CTL kill target cells to the same level while the ability of F5 Lck^{ind} OFF CTL to kill target cells was severely diminished. It is likely that in the absence of Lck, F5 CD8 T cells were not optimally activated in order to generate into fully functional CTL and therefore were unable to kill target cells. We have addressed this point further in **Chapter 5**. F5 WT and F5 Lck^{ind} ON CTL displayed similar levels of target cell lysis at the highest peptide dose, however as the peptide concentration decreased a difference between them was observed. None of the F5 CTL populations were able to kill the very lowest peptide pulsed target cells, suggesting that the antigen concentration was below the lower limit that can be recognised by CTL. The amount of antigen presented by target cells can also determine the outcome of action by CTLs. This is consistent with observations in some tumour studies where it has been shown that reduced antigen expression by tumour cells due to the down-regulation of the HLA-class I leads to inefficient activation of CTL and escape of tumour cells from immune recognition leading to disease progression (Lou et al., 2005; Vitale et al., 2005).

Various studies have been carried to determine how many pMHC molecules are required to activate helper T cells or cytotoxic T cells. One such study has used a single-cell imaging approach and showed that helper T cells and CTLs

could detect even one agonist pMHC (Irvine et al., 2002; Purbhoo et al., 2004). In the case of CTLs, however 10 pMHC complexes were required to achieve full calcium release and immunological synapse formation, and only 3 pMHC molecules were required to initiate killing of a target cell (Purbhoo et al., 2004). It is therefore possible that all T cells will be able to detect a single agonist ligand, but there are different thresholds for making a decision with respect to eliciting an effector function. Where there are different thresholds, there may be differences in the signalling machinery, either in the concentration or location of key molecules or in their pattern of interactions.

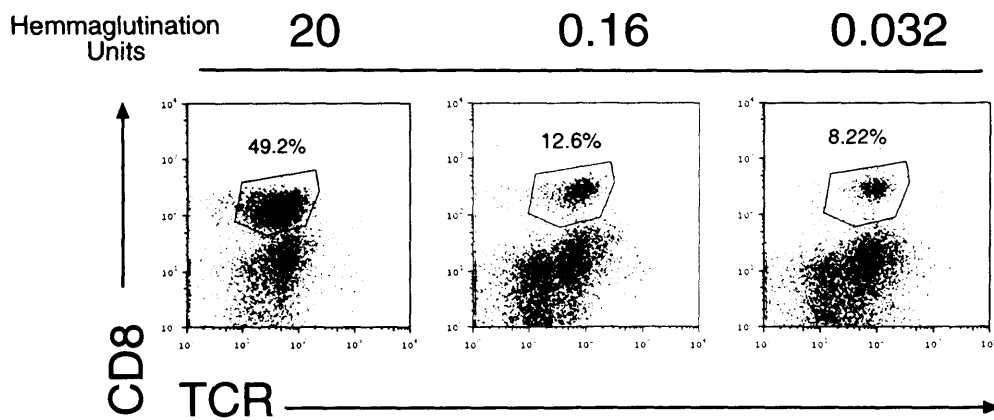
Since our experiments were conducted in lymphopenic host mice we wanted to assess the contribution of lymphopenia-induced proliferation compared with antigen-induced proliferation. A proportion of unimmunised F5 WT CD8 T cells recovered from the host mice upregulated CD44 and produced IFN γ . Our results are in agreement with studies, which have shown that when naïve T cells are adoptively transferred into lymphopenic host mice, they can proliferate independently of cognate antigen (Cho et al., 2000; Goldrath et al., 2004). Lymphopenia-induced proliferation of naïve T cells can also result in the upregulation of activation markers such as CD44 and in some cases the gaining of effector functions such as the ability to produce IFN γ and to lyse target cells, thus generating a memory like phenotype (Cho et al., 2000; Goldrath et al., 2004; Hamilton et al., 2006). A recent study by Hamilton et al has shown that CD8 T cells, which undergo lymphopenia-induced proliferation in the absence of CD4 T cells are unable to mediate protection against bacterial infection,

much like some conventional memory CD8 T cells generated in the absence of CD4 help (Hamilton et al., 2006). These memory like CD8 T cells were able to proliferate and were capable of differentiating into CTLs. They demonstrated that the expression of TRAIL, the apoptosis inducing ligand (Janssen et al., 2005) was defective, and interfered with other aspects of immunity such as clearing *L. monocytogenes* infection, via an unknown mechanism. We have also shown in our studies that F5 CTLs generated in the absence of Lck and F5 WT memory like CD8 T cells, displayed a similar phenotype. The number of cells recovered was similar and within each population a proportion of the cells upregulated CD44 expression, produced IFN γ upon restimulation *in vitro* and their ability to kill target cells was impaired. These cells were analysed 7 days after transfer, however the cell numbers and effector function for both groups had not improved 35 days post transfer (data not shown). Despite the similar phenotype and effector function observed in each of these groups, they were generated in different ways. The F5 WT memory like CD8 T cell effector function was generated in response to lymphopenia driven conditions in the absence of antigen. Whereas, F5 Lck^{ind} OFF CTL were generated in response to antigen. There may be a difference in the mechanism of differentiation between these two groups and one such difference will be discussed in **Chapter 5**. It would therefore be interesting to investigate the pathways of differentiation to elucidate the similarities and differences that lead to the development of this memory phenotype, which could be revealed by different gene expression patterns. One interesting candidate would be to measure the expression of TRAIL.

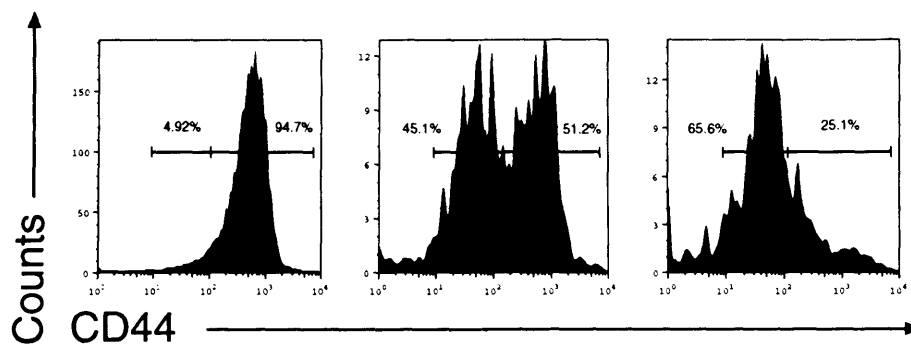
Figure 4.1: F5 CD8 T cells are efficiently activated with 20HA units of A/NT/60-68 influenza virus

2×10^6 F5 WT LN cells were transferred together with different titres of the influenza virus strain A/NT/60-68 A into Rag1^{-/-} host mice. Host mice were sacrificed after 7 days, and LN cells were stained with CD8 and TCR antibodies to determine the proportion of F5 WT CD8 T cells recovered. The cells were analysed by flow cytometry and a minimum of 10,000 total events were collected. (A) FACS plots show the F5 WT CD8 T cell recoveries and CD44 cell surface expression for mice immunised with 20, 4 and 0.032 HA Units of the influenza virus. (B) The graph shows the total number of F5 CD8 T cells recovered for each titre of virus.

A



Gated CD8 +



B

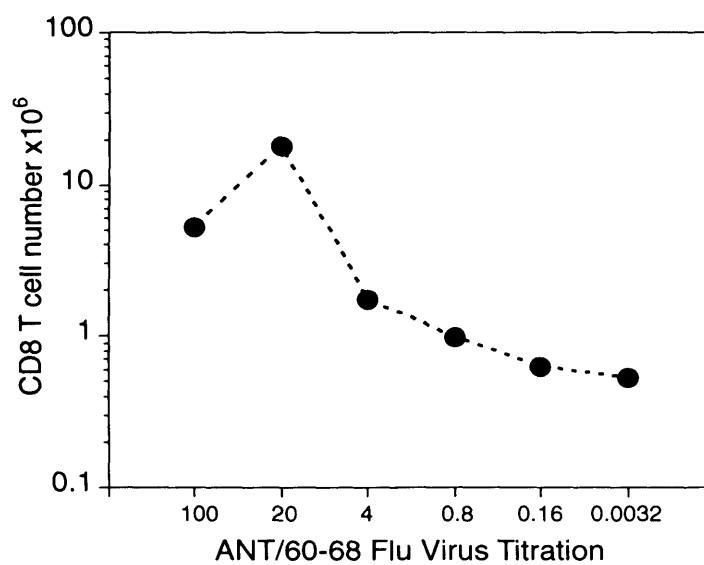
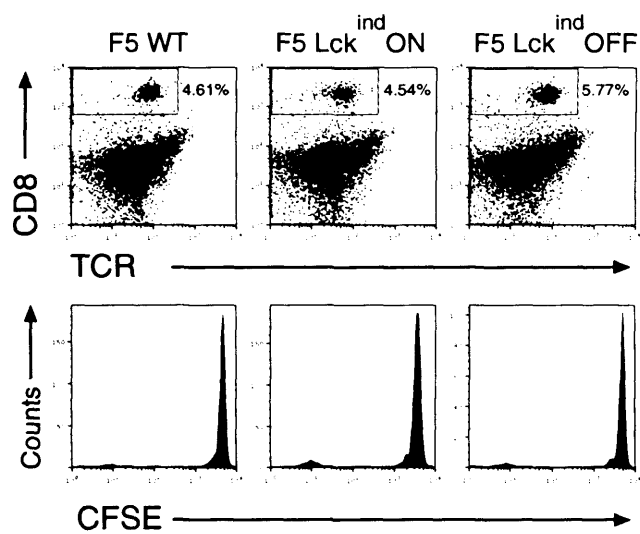


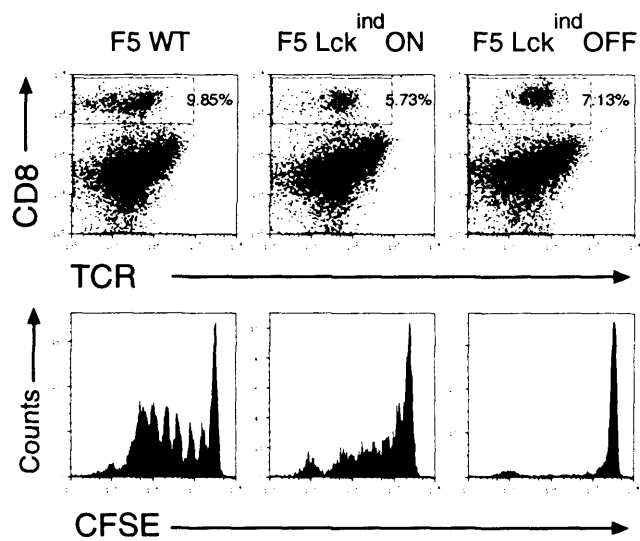
Figure 4.2: Early activation and division is defective in the absence of Lck

3×10^6 F5 CD8 T cells were labelled with CFSE and transferred together with A/NT/60-68 influenza virus into Rag1^{-/-} host mice. The mice were sacrificed at day 1 (A), and day 3 (B) and LN cells were stained with for CD8 and TCR. (A & B) CFSE profiles are shown for each group gated on CD8 T cells. (C) The graph shows the mean \pm SD percentage of F5 CD8 T cells which have undergone division at day 3, through analysis of their CFSE dilution. (D) The graph shows the number of divisions undergone by F5 CD8 T cells by day 3. The data show results for 3 mice per group and is representative of 2 independent experiments.

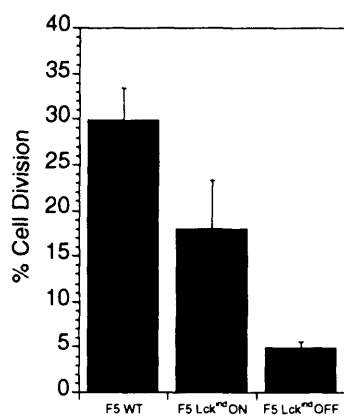
A Day 1



B Day 3



C



D

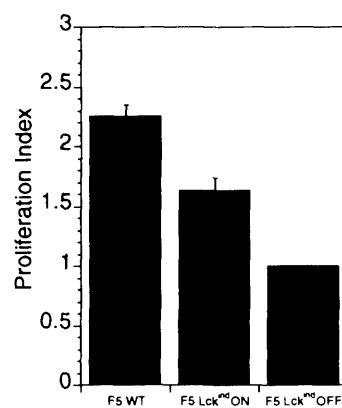
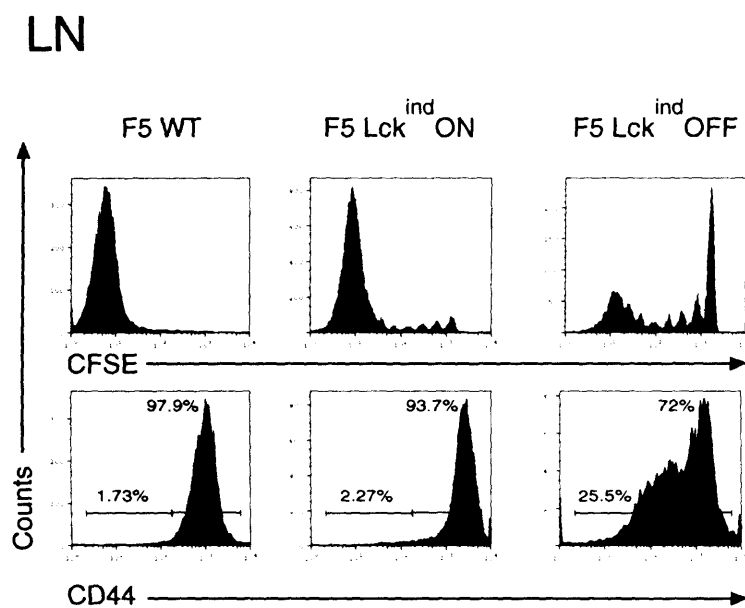


Figure 4.3: Reduced in expansion of activated F5 CD8 T cells is observed in the absence of Lck

3x10⁶ F5 CD8 T cells were labelled with CFSE and transferred together with A/NT/60-68 influenza virus into Rag1^{-/-} host mice. The mice were sacrificed at day 8 and LN and spleen cells were harvested. (A) FACS plots show the CFSE dilution of activated F5 CD8 T cells at day 8 and the cell surface expression of CD44 gated on CD8 positive T cells from the LN. (B) The graph shows the mean \pm SD number of total (LN and Spleen) CD8 T cells recovered for each group at days 1, 3 and 8 post transfer. The data show results for 3 mice per group and is representative of 2 independent experiments. Statistical significance was calculated using the Mann Whitney test (P< 0.05 = * P< 0.01 = **)

A



B

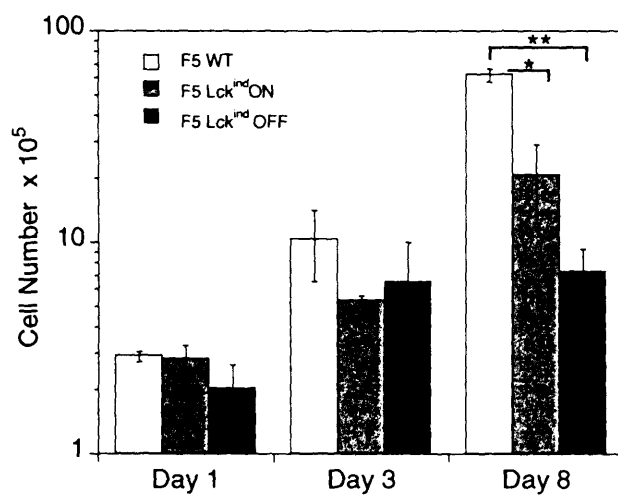
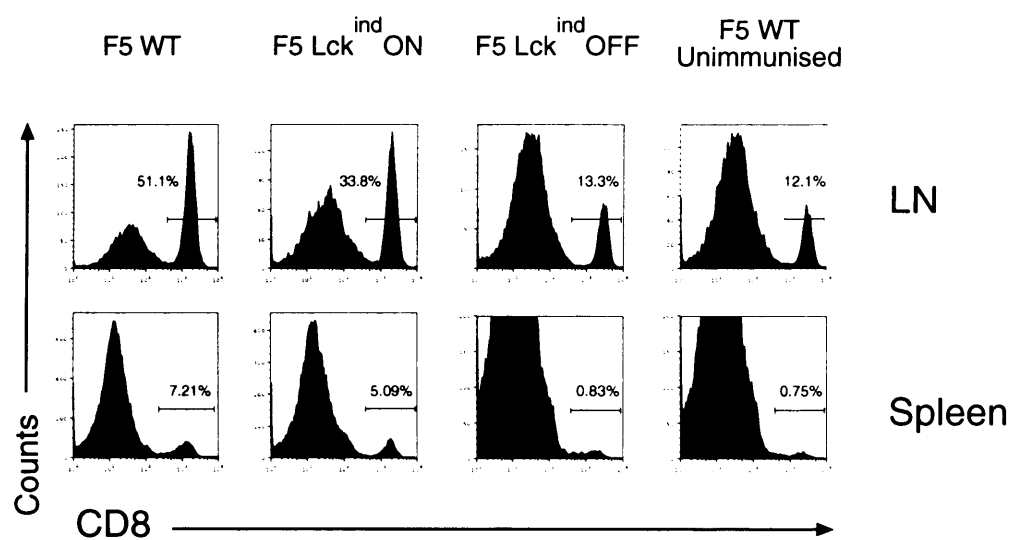


Figure 4.4: Poor expansion is observed in the absence of Lck in F5 CD8 T cells

F5 CD8 T cells were recovered from the LN and spleen of Rag1^{-/-} host mice and were stained for CD8 and CD45.1 to identify F5 CD8 T cells from the targets cells. The cells were analysed by flow cytometry, and a minimum of 10,000 total events were collected. (A) FACS plots show the proportion of F5 CD8 T cells recovered from the LN and spleen, the target cells were gated out in these profiles to enable clear identification of the F5 CD8 T cell populations. (B) The graph shows the mean (n=5 mice) \pm SD F5 CD8 T cell recoveries from the LN and spleen for each group. The data show results for 5 mice per group and is representative of 5 independent experiments. Statistical significance was calculated using the Mann Whitney test ($P < 0.01 = **$)

A



B

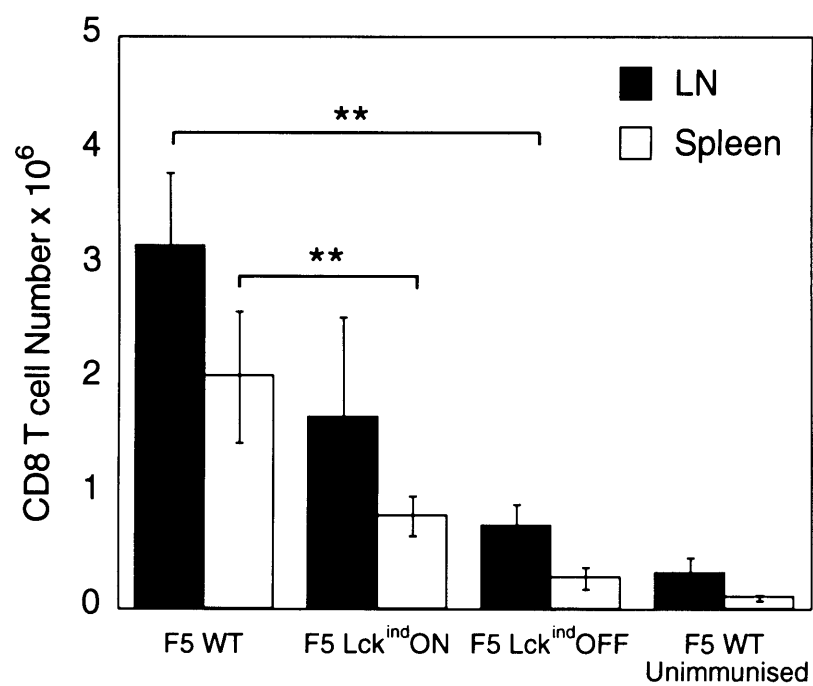
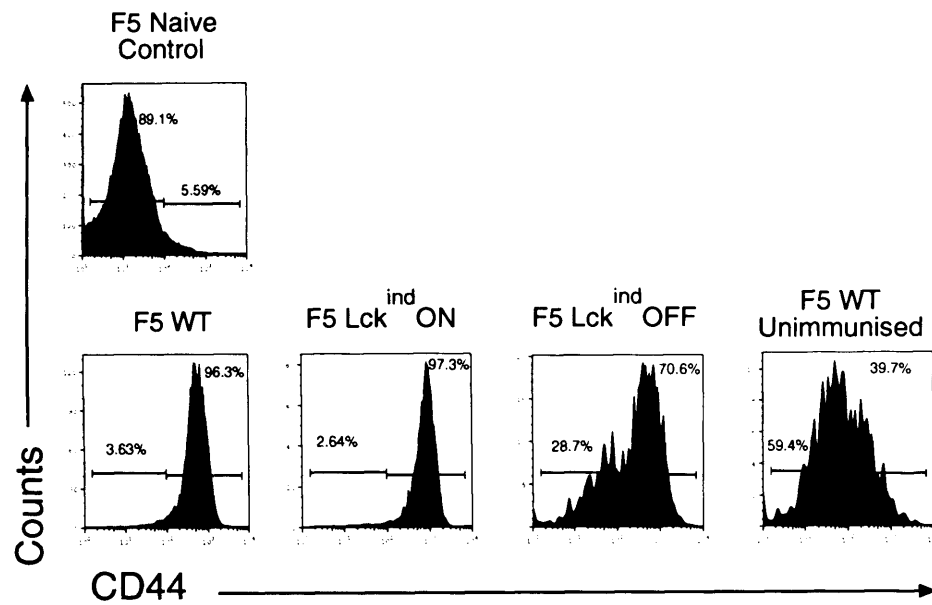


Figure 4.5: Defective CD44 up-regulation is observed in the absence of Lck

Cell surface expression of CD44 was measured by staining F5 CD8 T cells from the LN and spleen. The cells were analysed by flow cytometry and a minimum of 10,000 total events were collected. FACS plots show the cell surface expression of CD44 on F5 CD8 T cells from the LN (A) and spleen (B) gated on the CD8 T cells. The data show representative results for 5 mice per group and is also representative of 5 independent experiments.

A

LN



B

Spleen

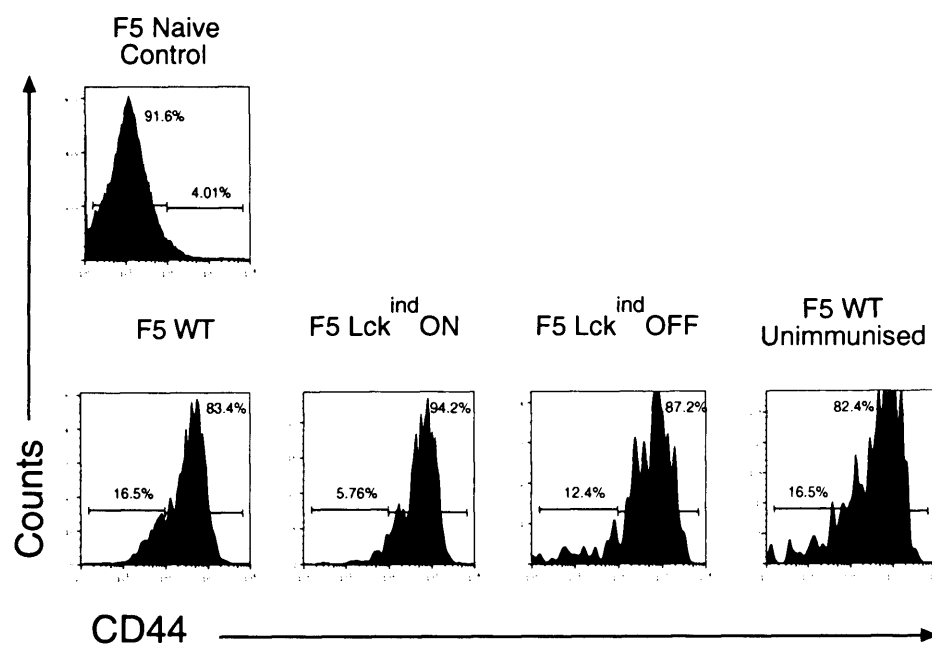
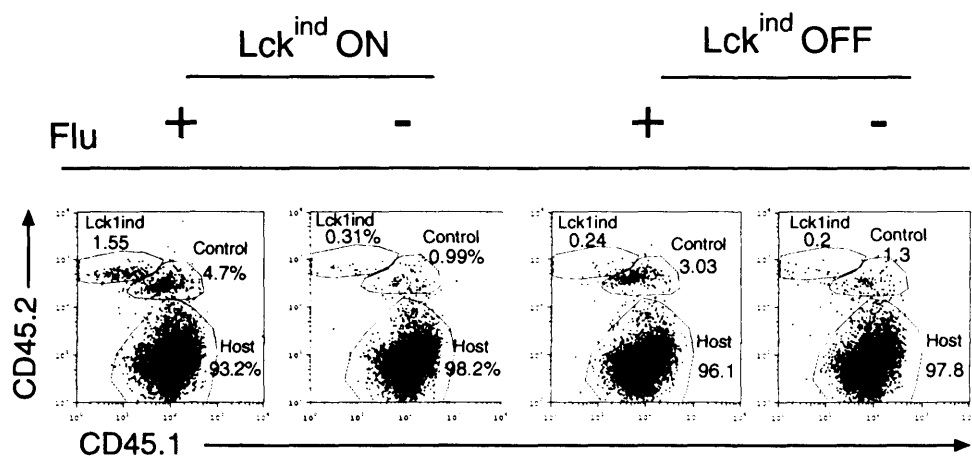


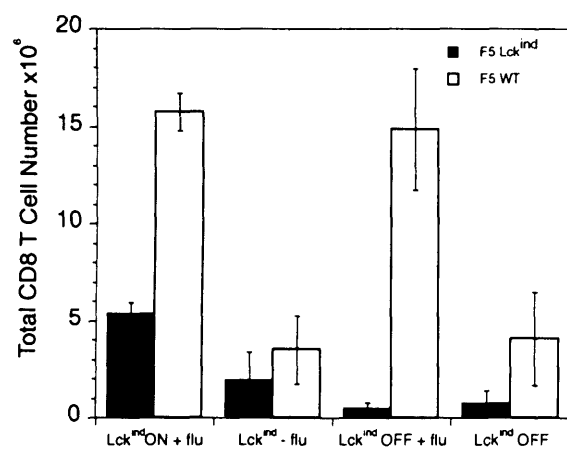
Figure 4.6: Activation and expansion of F5 CD8 T cells in the absence of Lck is defective upon transfer into intact hosts

F5 WT CD45.1/2, F5 Lck^{ind} ON and F5 Lck^{ind} OFF LN cells were transferred into intact tolerant host mice and immunised with the influenza A/NT/60-68 virus. Mice were sacrificed 7 days after transfer and LN cells were harvested and were stained for CD45.1 and CD45.2 to identify F5 WT control cells (CD45.1/2), F5 Lck^{ind} cells (CD45.2) and endogenous host cells (CD45.1) (A). The cells were analysed by flow cytometry and a minimum of 50,000 total events were collected. (B) The graph shows the mean \pm SD number of total F5 CD8 T cells recovered from each group of mice which were immunised or not with the influenza virus. (C) Histogram overlays showing CD44 cell surface expression on gated CD8 T cells of F5 WT and F5 Lck^{ind} cells (thin line) over endogenous host cells (shaded), each population has been normalised to 100%. The results show data for 5 mice per group and are representative of 2 independent experiments.

A



B



C

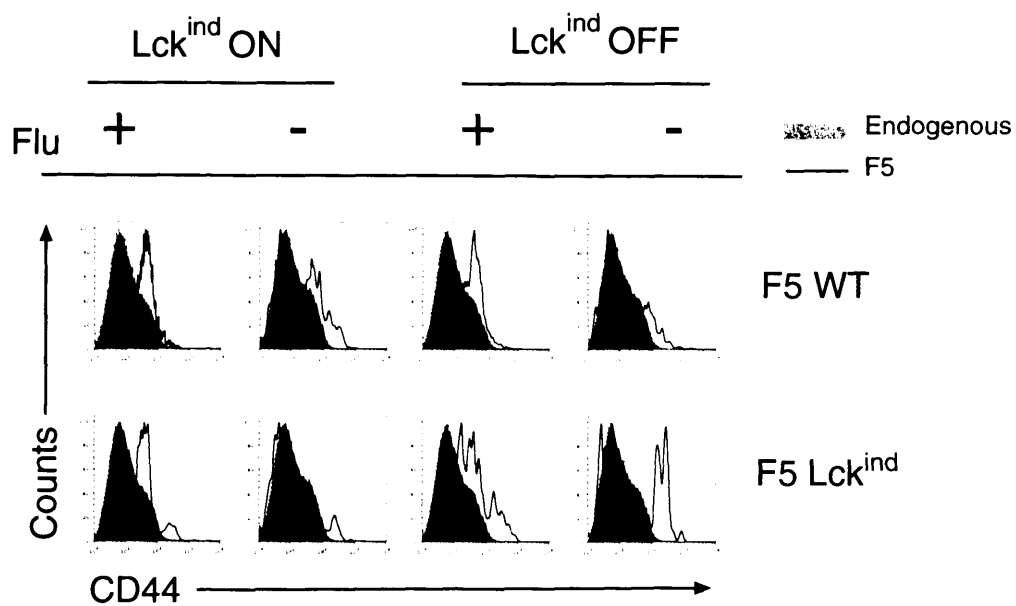
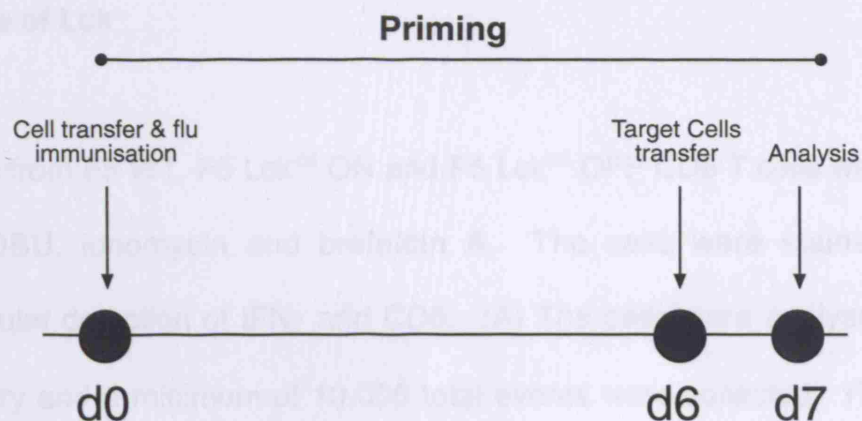


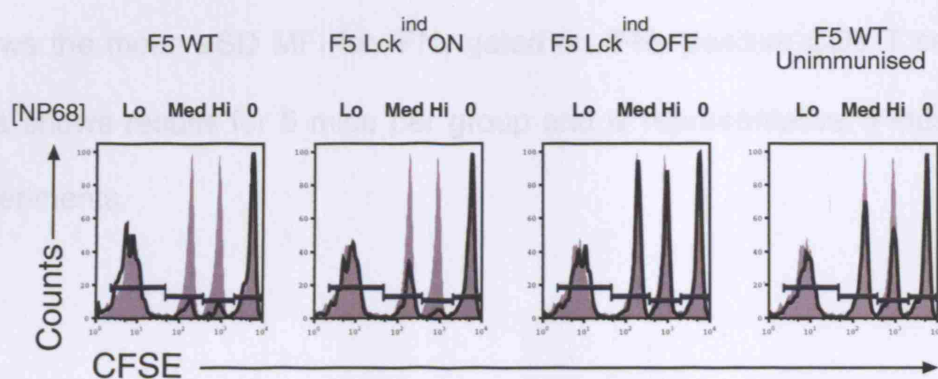
Figure 4.7: Lck is required for the cytolytic activity of effector F5 CD8 T cells *in vivo*

3×10^6 F5 CD8 T cells together with the influenza A/NT/60-68 virus were transferred into Rag1^{-/-} host mice at day 0. (A) Target cells were transferred into experimental mice overnight on day 6, and mice were sacrificed and analysed at day 7. LN derived target cells from C57BL/10/CD45.1 mice were pulsed with different concentrations of NP68 and labelled with different amounts of CFSE (0 NP68 + 1.25 μ M CFSE (0); 2.5×10^{-6} μ M NP68 + 0.25 μ M (Hi) CFSE; 2.5×10^{-9} μ M NP68 (Med) + 0.05 μ M CFSE and 2.5×10^{-12} μ M NP68 + 0 CFSE (Lo)). Targets cells were mixed in equal numbers and transferred into the Rag1^{-/-} host mice and naïve F5 Rag1^{-/-} control mice. (B) Histograms of CFSE expression profiles gated on target cells (CD45.1) were superimposed upon profiles of target cells from the naïve F5 Rag1^{-/-} control group (grey solid) over target cells recovered from the Rag1^{-/-} host group (black line), to show NP68 specific lysis by F5 CTL. (C) The graph shows NP68 specific lysis (Calculated as described in Methods and Materials) of target cells loaded with different concentrations of peptide by F5 CTL. Data shows results for 5 mice per group and is representative of 5 independent experiments.

A



B



C

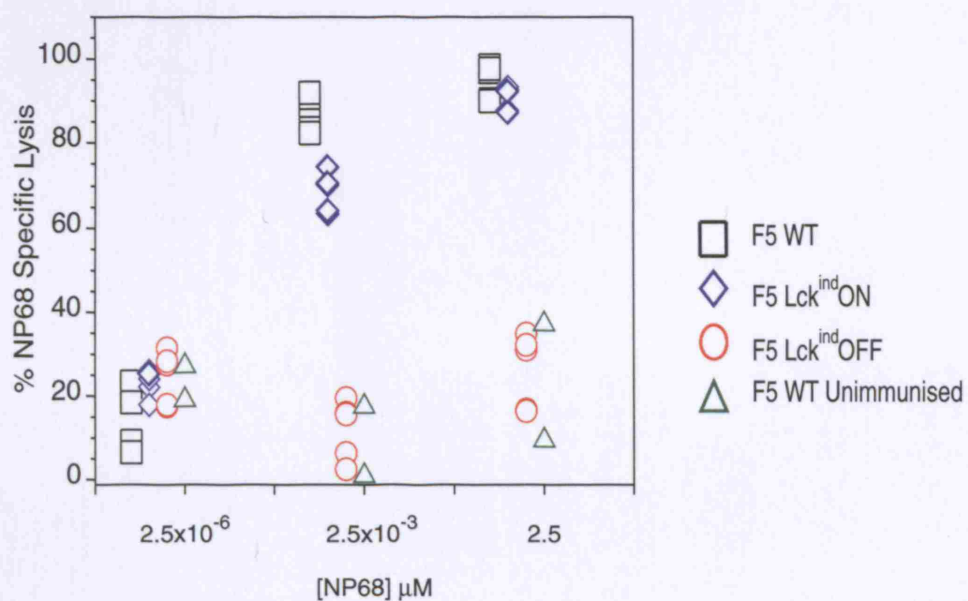
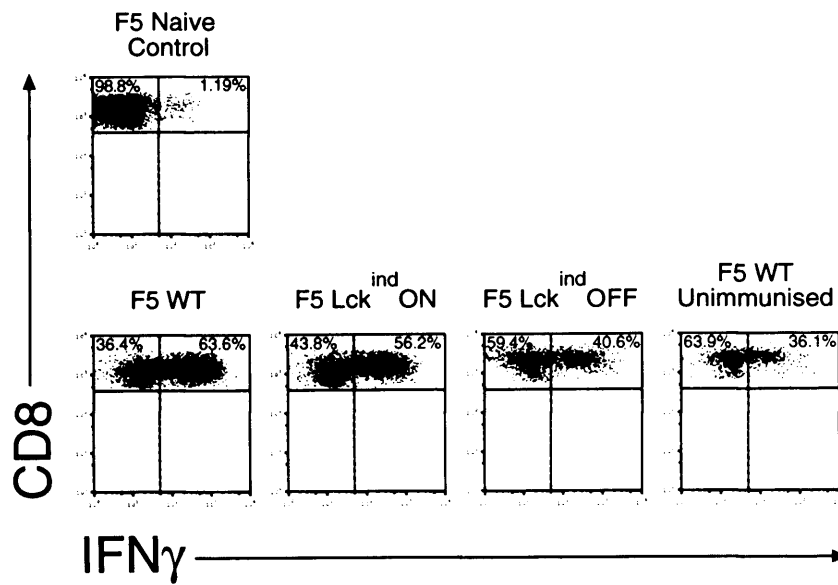


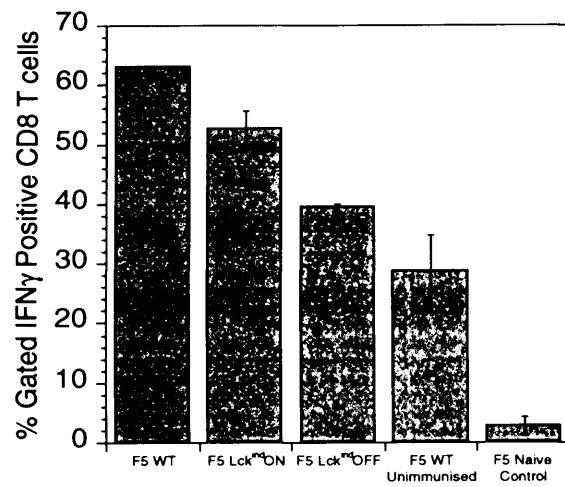
Figure 4.8: Reduced numbers of effector CD8 T cells produce IFN γ in the absence of Lck

LN cells from F5 WT, F5 Lck^{ind} ON and F5 Lck^{ind} OFF CD8 T cells were treated with PDBU, ionomycin and brefeldin A. The cells were stained for the intracellular detection of IFN γ and CD8. (A) The cells were analysed by flow cytometry and a minimum of 10,000 total events were collected. FACS plots show IFN γ positive F5 CD8 T cells. (B) The graph shows the mean \pm SD proportion of gated IFN γ positive F5 CD8 T cells are shown. (C) The graph shows the mean \pm SD MFI for IFN γ gated on IFN γ positive CD8 T cells. The data shows results for 5 mice per group and is representative 5 independent experiments.

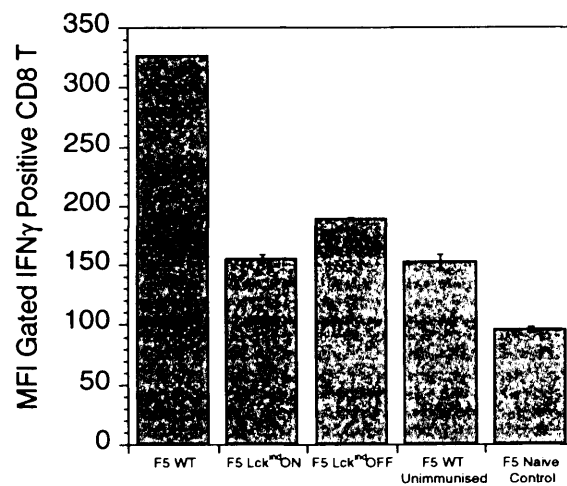
A



B



C



Chapter 5: The role of Lck in the generation and maintenance of Memory CD8 T cells.

5.1: Introduction

The CD8 T cell response can be divided into 3 distinct phases (Kaech et al., 2002b). The first stage is the expansion phase and is initiated in lymphoid organs, where encounter with antigen induces naïve CD8 T cells to clonally expand and differentiate into CTL (Butz and Bevan, 1998; Murali-Krishna et al., 1998). Following pathogen clearance, a contraction phase ensues in which greater than 90% of CTLs die via apoptosis. The surviving cells then enter the third stage, the memory phase, where a small fraction emerges and persists as memory cells. Memory CD8 T cells remain in a quiescent state, however upon re-activation by cognate antigen, they mediate a robust response defined by the rapid production of cytokines and the mediation of cytolytic function (Lau et al., 1994).

Many mechanisms have been proposed to be involved in the generation of memory CD8 T cells. One hypothesis is that memory T cells have increased TCR signal transduction, increasing their responsiveness to antigen (Kersh et al., 2003). Various studies have been conducted to elucidate whether TCR signalling is different in naïve, effector and memory T cells. Early studies with memory CD4 T cells demonstrated the increased responsiveness to antigen recall was because of alterations in the TCR signal transduction machinery

(Farber et al., 1997; Farber et al., 1995). Studies focusing on memory CD8 T cells reported increased Lck localisation to the CD8 co-receptor (Bachmann et al., 1999b), and an elevated level of Lck expression (Kaech et al., 2002a; Slifka and Whitton, 2001). It has also been shown that the increase in phosphorylated tyrosine residues in lipid rafts was higher in memory CD8 T cells than in naïve T cells and more specifically the increased responsiveness of effector and memory CD8 T cells was associated with higher levels of Lck (Kersh et al., 2003). Tewari et al have also addressed the requirement of Lck signalling in memory CD8 T cells, their studies concluded that Lck expression was not required for the activation and effector function, such as the production of IFN γ , of memory CD8 T cells (Tewari et al., 2006). Here we examine the contribution of Lck signalling at different stages of memory F5 CD8 T cell generation and the maintenance of the memory compartment, and the ability of memory F5 CD8 T cells to mediate cytolytic activity and produce IFN γ . In contrast to Tewari et al we report that Lck has differential requirements in mediating effector functions in memory CD8 T cells. We show that Lck is required for memory F5 CD8 T cells to kill target cells, but the requirement for Lck in IFN γ production upon re-stimulation was less stringent.

5.2: Generation of an F5 Memory CD8 T cell Population

To address the role of Lck in the generation of memory CD8 T cells, F5 CD8 T cells were transferred into Rag1^{-/-} host mice together with the influenza A/NT/60-68 virus, as shown by the scheme in **Figure 5.1A**. For memory CD8 T cells, cells were analysed at day 35 or later, since after day 7 cells will have

undergone proliferation and are likely to have generated a resting memory phenotype. Firstly we wanted to establish if Lck expression affected the number of F5 CD8 memory T cells generated. **Figure 5.2** shows the proportion **(A)** and number **(B)** of F5 CD8 T cells recovered from the LN and spleen of the recipient mice. There were a higher number of CD8 T cells recovered from the LN compared with the spleen in each group. CD8 T cell numbers recovered from the F5 Lck^{ind} ON mice were lower compared with the F5 WT, and in the F5 Lck^{ind} OFF mice CD8 T cell number was significantly reduced further (**Figure 5.2B**). The recovery of F5 CD8 T cells 5 weeks post transfer was similar to the number of effector F5 CD8 T cells recovered after 7 days (**Figure 4.4**). This data suggest by day 7, cell proliferation had stopped, and by day 35 resting memory F5 CD8 T cells were generated.

We measured the cell surface expression of the activation marker CD44 and also the effector and memory cell markers CD62L and CD43 (**Figure 5.3**) (Kaeche et al., 2002b; Oehen and Brduscha-Riem, 1998; Onami et al., 2002). Memory F5 CD8 T cells from each group showed upregulation of CD44 expression in both the LN and spleen (**Figure 5.3A**). CD62L is expressed on central memory CD8 T cells (Kaeche et al., 2002b; Oehen and Brduscha-Riem, 1998), and majority of the F5 WT and Lck^{ind} ON CD8 T cells expressed CD62L, however in the F5 Lck^{ind} OFF group fewer cells expressed CD62L (**Figure 5.3B**). We also measured the cell surface expression of the activation-associated glycoform of the protein CD43, whose glycosylation pattern is altered upon T cell activation (Onami et al., 2002) (**Figure 5.3C**). Approximately

9% of F5 WT and Lck^{ind} ON CD8 T cells expressed CD43, whereas fewer cells expressed CD43 in F5 Lck^{ind} OFF samples (**Figure 5.3C**). CD44 is the most reliable marker for memory cells, and we showed CD44 expression was the same for each group. In addition we measured the expression of CD62L and CD43 and some small differences were observed, overall however, F5 Lck^{ind} OFF CD8 T cells appeared to generate a memory phenotype (CD44^{Hi}, CD62L⁺, CD43⁻) like the F5 WT and F5 Lck^{ind} ON CD8 T cells. Also there appears to be a good correlation between the CD62L expressing and the CD43 negative F5 CD8 T cells, suggesting the cells have a central memory phenotype rather than effector phenotype.

5.3: Lck is required for the cytolytic function of memory CD8 T cells

Lck expression is required for the generation and mediation of effector function of F5 CTL as described in Chapter 4. We wanted to further address if Lck was required for eliciting effector function of F5 memory CD8 T cells. F5 CD8 T cells were transferred into Rag1^{-/-} recipient mice and challenged with the influenza A/NT/60-68 virus, followed after 34 days by target cells to test the cytolytic function of the F5 memory CD8 T cells (**Figure 5.1A**). The data shows F5 Lck^{ind} OFF memory CD8 T cells had significantly impaired killing of targets cells pulsed with a high dose of peptide compared with F5 WT and F5 Lck^{ind} ON memory CD8 T cells (**Figure 5.4 A & B**). Target cell lysis by F5 Lck^{ind} ON memory CD8 T cells was slightly lower than the F5 WT memory CD8 T cells for targets pulsed with the highest dose of peptide and considerably lower for targets pulsed with an intermediate dose of peptide. At the lowest dose of

peptide neither F5 WT, F5 Lck^{ind} ON nor F5 Lck^{ind} OFF memory CD8 T cells were able to mediate target cell killing. These data support the findings in **Chapter 4**, where we showed the amount of antigen presented by target cells, in combination with the level of Lck expression in F5 CD8 T cells, appears to determine the threshold for activating effector cells to mediate target cell lysis. Here we show the same is true for memory cell generation and function.

5.4: Number of IFN γ producing CD8 T cells is reduced in the absence Lck expression

We also measured the capacity of F5 memory CD8 T cells to produce IFN γ upon stimulation *in vitro* (**Figure 5.5**). Approximately 70% of F5 WT memory CD8 T cells produced IFN γ upon restimulation *in vitro*, whereas F5 Lck^{ind} ON memory CD8 T cell showed a reduced capacity (~40%) to produce IFN γ compared with F5 WT memory CD8 T cells (**Figure 5.5A**). The percentage and number of IFN γ producing memory CD8 T cells was further decreased in F5 Lck^{ind} OFF mice to ~25% (**Figure 5.5A & B**). However the amount of IFN γ production per cell, indicated by the MFI, for F5 Lck^{ind} ON and F5 Lck^{ind} OFF memory CD8 T cells was comparable and this was reduced compared to F5 WT memory CD8 T cells (**Figure 5.5C**). Tewari et al showed in their system Lck was not required for memory CD8 T cells to produce IFN γ upon restimulation *in vitro*. However they have made comparisons between Lck^{ind} ON and Lck^{ind} OFF memory CD8 T cells, which we also show to be similar. Had they compared responses to memory CD8 T cells expressing WT levels of Lck, there may well be a difference, which was not uncovered in their study (Tewari

et al., 2006). In agreement with the data presented in **Chapter 4** we have shown that the requirement for Lck expression in F5 memory CD8 T cells was less stringent for the production of IFN γ upon re-stimulation compared to the cytolytic function.

5.5: A small component of CTL kill target cells via the Fas/FasL pathway and is dependent on Lck expression

We have shown that memory F5 CD8 T cells generated in the absence of Lck show defective lysis of target cells compared to the Lck expressing controls. However some killing above background was generally detected from the F5 Lck^{ind} OFF CD8 T cells. We wanted to address if the mechanism by which F5 WT CD8 T cells mediate target cell lysis was different in Lck deficient memory F5 CD8 T cells. Two of the most common mechanisms by which CTL mediate target cell lysis is through either the perforin/granzyme or Fas/FasL mediated pathways (Topham et al., 1997). We therefore compared killing of target cells that were WT or from *lpr* which are deficient for Fas and therefore mice only susceptible to perforin mediated killing. Target cells were labelled with different concentrations of CFSE to identify pulsed and unpulsed WT and *lpr* peptide populations and injected together into recipient mice containing memory cells (**Figure 5.6A**). FACS plots show the specific lysis of WT and *lpr* targets cells by F5 WT, F5 Lck^{ind} ON and F5 Lck^{ind} OFF memory CD8 T cells (**Figure 5.6B & C**). Both F5 WT and F5 Lck^{ind} ON memory CD8 T cells were able to kill *lpr* target cells but at a reduced level compared to their ability to kill WT target cells. This indicates that Fas mediated killing is not the major contributor to killing of target

cells. Killing by F5 Lck^{ind} OFF memory CD8 T cells was lower for both WT and *lpr* targets cells, indicating that a small component of this killing may be Fas dependent, however the difference is not significant (**Figure 5.6C**). FasL expression was also assessed and was found to be reduced on memory CD8 T cells in the absence of Lck, compared with F5 WT and F5 Lck^{ind} ON memory CD8 T cells (**Figure 5.6D**). Early studies demonstrated that Lck could control the expression of FasL (Gonzalez-Garcia et al., 1997), and so F5 Lck^{ind} OFF memory CD8 T cells have reduced expression of FasL, which could explain their diminished ability to kill via the Fas pathway. The data showed that a small component of Lck expressing F5 memory CD8 T cells mediate target cell lysis via the Fas/FasL pathway however F5 Lck^{ind} OFF memory CD8 T cells lose both the ability to kill through perforin and Fas.

The data in **Chapters 3 & 4** demonstrate the requirement for signals through Lck in the triggering and expansion of activated F5 CD8 T cells. *In vivo* the numbers of F5 CD8 T cells with a memory (CD44 hi) phenotype recovered in the absence of an Lck signal were also diminished, and furthermore these cells display defective CTL effector function. Given that the absence of Lck compromised primary activation of the cells it is unclear whether they had differentiated fully into memory cells. From these data we were unable to differentiate between a requirement for Lck in the generation of F5 CD8 memory as opposed to the maintenance of memory F5 CD8 T cells once they have been generated. To resolve this, we asked whether removal of Lck from

optimally generated F5 memory CD8 T cells, impaired their survival and cytolytic activity.

5.6: Lck is not required for the survival of memory CD8 T cells

Early work has shown that TCR signals are required for the survival of the naïve peripheral T cell pool, but were not required for the survival of CD4 memory T cells (Seddon et al., 2003). Previous studies have also shown that memory CD8 T cells are able to persist in the absence of MHC, suggesting that TCR-MHC contact is not required for their maintenance and survival (Murali-Krishna et al., 1999). However contrasting studies showed that upon TCR ablation *in vivo*, TCR-deficient memory CD8 T cell numbers declined over time (Polic et al., 2001), suggesting some component of TCR signalling was required for their survival.

To this end we addressed the contribution of signalling by Lck in the maintenance and survival of memory F5 CD8 T cells. To assess the role of Lck in memory CD8 T cell survival, Lck expression was maintained during the initial stages of CD8 T cell priming. 7 days post cell transfer at the peak of the response Lck expression was switched off by the removal of dox from the feed of the mice and for comparison in a second group Lck expression was switched off after 4 weeks following memory formation (The experimental protocol is illustrated in **Figure 5.1B**). Similar proportions of F5 CD8 T cells were recovered from the F5 Lck^{ind} ON groups, in which Lck was maintained throughout the duration of the experiment, and from the groups of mice where

Lck expression was switched off 7 days and 28 days post transfer (**Figure 5.7A & B**). From these data we can conclude that maintenance of F5 CD8 memory T cells does not require Lck expression. Our data is in agreement with recent studies conducted by Tewari et al who have also demonstrated that Lck is not required for the maintenance of virus specific memory CD8 T cells (Tewari et al., 2006). It is not possible to say whether lack of requirement for Lck equates to lack of requirement for TCR signalling since in the absence of Lck, Fyn has been shown to play a role in various T cells responses, for example in T cell development (Groves et al., 1996; van Oers et al., 1996) and the survival of the naïve T cell compartment, where it was demonstrated that expression of either Lck or Fyn was required for survival (Seddon and Zamoyska, 2002b). Our data show that Lck is not required for the survival of F5 memory CD8 T cells, however we would need to investigate further whether Fyn is able to compensate for the absence of Lck, or whether Fyn has a unique role in the maintenance of memory CD8 T cells.

We analysed the cell surface expression patterns of CD44, CD62L and CD43 of the recovered F5 CD8 T cells to ascertain if the memory phenotype was altered by switching Lck expression off at the different time points following priming. **Figure 5.8** shows similar CD44 (**A**), CD62L (**B**) and CD43 (**C**) expression patterns in each F5 Lck^{ind} group irrespective of Lck expression after the priming phase. The data shows that F5 CD8 T cell maintenance of this memory phenotype does not require continuous Lck expression following memory F5 CD8 T cell generation.

5.7: Switching Lck expression off reduces the cytolytic function of F5 CD8 memory T cells

F5 CTL generated in the absence of Lck were not able to mediate effective cytolytic function *in vivo* (**Figure 4.6**), showed reduced levels of granzyme B production (**Figure 3.8B**) and their expansion was also significantly impaired (**Figure 4.3 & 4.4**). In principal the defective killing could be explained either by the lack of expansion or by impaired formation of CTL due to a defect in the triggering of effector F5 CD8 T cells in the absence of Lck or a combination of the two. To address these questions, we looked at the killing ability of the groups in which we switched Lck expression off following priming at day 7 and after 28 days (**Figure 5.9A**). Target cells were transferred into the host mice overnight on day 34. As before F5 WT memory CD8 T cells demonstrated high levels of target cell lysis, and F5 Lck^{ind} ON memory CD8 T cells showed reduced lysis compared to the F5 WT mice. When Lck expression was switched off after 7 days or 28 days post transfer we observed a further reduction in target cell lysis compared to the F5 Lck^{ind} ON group (**Figure 5.9B and C**), despite the fact that the numbers of F5 CD8 cells recovered from these groups of animals were equivalent (**Figure 5.7**) and their effector phenotypes were identical. These data show that expression of Lck substantially influences the lytic ability of the cells and therefore, the primary defect of these cells is their inability to kill target cells in the absence of Lck.

When we measured IFN γ production we show that the percentage of IFN γ producing CD8 T cells was similar between the F5 Lck^{ind} groups despite

switching Lck expression off at either day 7 or day 28 (**Figure 5.10A and B**). However the amount of IFN γ production per cell was reduced in the groups in which Lck was turned off compared to the F5 WT and F5 Lck^{ind} ON memory CD8 T cells (**Figure 5.10C**). in contrast to the data presented in **Figure 5.5C** in this experiment we see the amount of IFN γ produced by F5 Lck^{ind} ON memory CD8 T cells was equivalent to the F5 WT groups. However in general there was a reduced production of IFN γ in cells with low level of Lck expression compared with the WT group, but this was not observed for this particular experiment.

We have shown that Lck contributes to multiple stages of memory cell formation and is indeed required for the priming, expansion and differentiation of F5 CD8 T cells but is not required for the survival of memory F5 CD8 T cells.

5.8: Re-expression of Lck in F5 memory CD8 T cells generated in the absence of Lck restores lytic function.

We have shown that Lck is required for eliciting cytolytic function of both effector and memory F5 CD8 T cells. We wanted to address if we could restore lytic function of memory CD8 T cells generated in the absence of Lck by switching Lck expression back on and testing the killing capacity. This experiment would address whether differentiation of the lytic machinery had occurred in the absence of Lck. We transferred F5 CD8 T cells into Rag1^{-/-} host mice and immunised them with the influenza A/NT/60-68 virus. Two groups of F5 Lck^{ind} OFF groups were set up in which Lck expression was restored in one

group by feeding the host mice dox 28 days after transfer and activation, see scheme in **Figure 5.11A**. In another group F5 Lck^{ind} ON CD8 T cells were transferred and Lck expression was switched off after 7 days and switched back on 7 days before target cells were introduced. This combination of switching Lck on and off leads to some clear conclusions about the role of Lck in generating CD8 memory and effector function. First, Lck is required for expansion in response to antigen as the groups in which an Lck signal was maintained during priming showed comparable cell recoveries after 5 weeks (**Figure 5.11B**). In contrast the F5 Lck^{ind} OFF groups showed a marked reduction in CD8 T cell recoveries compared with all groups in which Lck expression was on during the priming phase.

Second we tested the cytolytic function of F5 CD8 memory T cells in the presence or absence of Lck expression. If Lck is on at the time of the CTL assay, good levels of target killing are seen (**Figure 5.11C**). F5 Lck^{ind} ON CD8 T cells in which Lck expression was switched off after 7 days (F5 Lck^{ind} ON OFF d7, yellow diamond) showed reduced cytolytic function compared with the F5 Lck^{ind} ON (blue diamond) group in which Lck expression was maintained throughout the experiment (**Figure 5.11C**). Remarkably, the group in which Lck expression was switched off after 7 days and switched back on shortly before the assay (F5 Lck^{ind} ON OFF d7 ON d28, purple diamond) demonstrated cytolytic function similar to the F5 Lck^{ind} ON group. Interestingly F5 Lck^{ind} OFF memory CD8 T cells generated in the absence of Lck in which Lck was subsequently switched back on 7 days prior to target cell transfer (F5 Lck^{ind}

OFF ON d28, red circle) showed a significant increased ability to kill target cells compared with the F5 Lck^{ind} OFF group (green circle). The cytolytic function in this group was restored comparable to F5 Lck^{ind} ON levels showing the lack of killing was reversible. Given the cell recovery in this group was reduced on a per cell basis, the cytolytic function of these F5 memory CD8 T cells was more efficient than the groups in which Lck expression was maintained throughout the duration of the experiment. One possible explanation of this result is that antigen was still present in the system and could stimulate cells once Lck expression was switched back on resulting in the proliferation and differentiation of the cells at this point. This could explain the ability of these memory F5 CD8 T cells to kill target cells to the same extent as the F5 Lck^{ind} ON memory CD8 T cells. However this was unlikely, as one would expect that contact with antigen after switching Lck expression on would result in cell proliferation, and we recovered the same number of F5 memory CD8 T cells in this group as in the F5 Lck^{ind} OFF group suggesting no additional proliferation occurred. The data therefore suggests that antigen was not present at this point, and the re-expression of Lck per se was sufficient to restore cytolytic function in F5 Lck^{ind} OFF memory CD8 T cells. To fully address this and confirm the absence of antigen, we would need to carry out further experiments to investigate the activation state of these cells.

In **Chapter 4** we showed that F5 Lck^{ind} OFF CD8 effector T cells and the F5 WT unimmunised group had generated a similar phenotype with respect to the expression of cell surface marker CD44, cell recovery and development of

effector function. It appears that there is a difference in the mechanism of differentiation between F5 Lck^{ind} OFF memory CD8 T cells which were generated in response to antigen and F5 WT memory like CD8 T cells generated in response to lymphopenia. As shown in **Figure 5.11** the re-expression of Lck in F5 Lck^{ind} OFF memory CD8 T cells restored cytolytic function to WT levels. By comparison F5 WT memory like CD8 T cells were unable to elicit cytolytic activity, despite expression of WT levels of Lck. To investigate the pathways of differentiation between these two groups at different stages, gene expression profiling will be a useful tool.

Finally to see if restoring Lck expression affected IFN γ production, we measured IFN γ secretion after stimulating memory F5 CD8 T cells *in vitro*. We showed the proportion of IFN γ positive cells was similar between all F5 Lck^{ind} ON groups and both F5 Lck^{ind} OFF groups (**Figure 5.12**). Once again the F5 WT memory CD8 T cells had an increased capacity to produce IFN γ compared with all the other groups (**Figure 5.12A and B**). In this experiment the MFI of IFN γ produced was also similar between the different groups in which Lck expression was either switched on or off. The F5 WT group produced higher levels of IFN γ in comparison to all the other F5 Lck^{ind} groups (**Figure 5.12C**).

5.9: CD4 T cells improve the expansion of activated CD8 T cells and generation of memory cells

Some studies have shown that CD4 T cells are required for memory CD8 T cell development and efficient reactivation of secondary responses (Bourgeois et

al., 2002b; Shedlock and Shen, 2003; Sun and Bevan, 2003). The experiments described here have been conducted in the absence of CD4 T cells, and showed that activated F5 Lck^{ind} OFF CD8 T cells were unable to produce IL-2 and to expand efficiently (**Figure 3.2 & 3.5C**). We therefore wanted to see if adding CD4 help would improve the expansion and effector function of activated F5 CD8 T cells and development of memory F5 CD8 T cells particularly in the absence of Lck, possibly providing a source of IL-2. We co-transferred polyclonal WT CD4 T cells together F5 CD8 T cells into Rag1^{-/-} recipient mice which were challenged with the influenza virus. F5 CD8 T cells were harvested from the LN and spleen of the recipient mice after 5 weeks (**Figure 5.13**). Co-transfer of WT CD4 T cells did not improve the expansion of F5 CD8 T cells recovered from the LN compartment of F5 WT mice after 5 weeks but seemed to increase recovery of cells in F5 Lck^{ind} ON and OFF mice (**Figure 5.13A**). However there was a significant increase in the numbers of F5 CD8 T cells recovered from the spleen in each group, therefore overall more cells were recovered (**Figure 5.13B**). The difference observed between the LN and spleen suggest that the presence of CD4 T cells encourage homing and expansion in the spleen, something which was not observed in mice in which F5 CD8 T cells were transferred in the absence of CD4 T cells. The presence of CD4 T cells may be providing additional cytokines and/or an improved microenvironment in the spleen, which would be more favourable for memory F5 CD8 T cells to reside. Also in this set of experiments we did not measure CD62L expression, which has been shown to be an important LN homing receptor (Gallatin et al., 1983; Oehen and Brduscha-Riem, 1998). It is therefore

possible that CD4 T cells are helping the priming of F5 CD8 T cells, resulting in more activated F5 CD8 T cells which could lead to the down regulation of CD62L expression leading to greater numbers of F5 memory CD8 T cells being recovered from the spleen.

We asked whether production of IL-2 by the CD4 T cells was directly responsible for the enhanced expansion of the F5 CD8 T cells in the same study by co-transferring IL-2 deficient polyclonal CD4 T cells with F5 CD8 T cells into the Rag1^{-/-} recipient mice. A similar number of CD8 T cells were recovered from the LN for the three groups transferred with IL-2 deficient CD4 T cells, however in the spleen a further increase in F5 CD8 T cell recovery was observed (**Figure 5.13B**). Analysis of the phenotype of memory F5 CD8 T cells recovered following transfer with WT CD4 T cells showed similar levels of CD44 upregulation and the proportion of CD43 expression was similar between groups and when compared to the groups, which did not receive any CD4 T cells (**Figure 5.14**). However F5 CD8 T cells which were co-transferred with IL-2 deficient CD4 T cells showed a population of cells, which had high CD43 cell surface expression, but had not upregulated CD44 expression. One possible explanation is that these cells with a naïve phenotype cells were not of F5 origin and it is possible that there could have been contamination of polyclonal CD8 T cells that contaminated the IL-2 deficient CD4 T cells preparation. This is possible because IL-2 deficient mice show lymphoproliferation. To address this we tried to identify the F5 CD8 T cells by measuring the expression of Vβ11

however the antibody was not working properly, so we were unable to address this point directly.

It is also possible that the presence of CD44 low CD8 T cells recovered from the recipient mice, which were co-transferred with IL-2 deficient CD4 T cells, could have been contaminating polyclonal CD8 T cells derived as result of de novo T cell development. Studies have shown that naïve T cells transferred into lymphopenic recipient mice generally undergo lymphopenia-induced proliferation and acquire a memory like phenotype, including the upregulation of CD44 (Cho et al., 2000; Goldrath et al., 2000). However two studies have demonstrated that approximately 8 weeks following naïve T cell transfer into lymphopenic recipient mice, a population of naïve phenotype T cells were recovered, which were CD44 low (Ge et al., 2002; Tanchot et al., 2002). One study suggested that the observation was due to naïve T cells transiently acquiring a memory like phenotype, and following repopulation of the periphery subsequently reverted back to a naïve state (Goldrath et al., 2000). However an alternative explanation for these observations was the presence of haematopoietic precursors in the spleen of adult mice (Hamad et al., 1995; Katsura et al., 1988) and the accumulation of donor derived naïve T cells could be from de novo T cell development in the thymus (Ge et al., 2002; Tanchot et al., 2002).

5.10: CD4 help enhances killing of Lck-sufficient but not Lck-deficient F5 memory CD8 T cells and this is dependent on IL-2

The presence of CD4 helper T cells improved the expansion of F5 CD8 T cells in response to influenza virus (**Figure 5.13**). We asked whether cytolytic function was similarly improved in the presence of CD4 help. Antigen pulsed target cells were transferred into the recipient mice after 5 weeks to test their ability to mediate cytolytic function. At the high peptide dose F5 WT memory CD8 T cells were able to mediate similar levels of target cell lysis irrespective of CD4 T cells (**Figure 5.15A**), however at the intermediate peptide dose F5 WT memory CD8 T cells generated in the presence of WT CD4 T cells showed improved killing of target cells (**Figure 5.15B**) which required the CD4 T cells to be able to make IL-2. F5 Lck^{ind} ON memory CD8 T cells also showed improved lytic ability with WT CD4 T cells both at high and intermediate peptide concentrations (**Figure 5.15**), but not with IL-2 deficient CD4 T cells, where the killing capacity was equivalent to those memory F5 CD8 T cells generated in the absence of any CD4 T cells. F5 Lck^{ind} OFF memory CD8 T cells did not demonstrate any improvement in target cell lysis in the presence of CD4 T cells at either dose of peptide. Furthermore co-transfer with IL-2 deficient CD4 T cells made no difference to the lytic ability of memory CD8 T cells generated in the absence of Lck, which was low in all three groups. Given that the numbers of CD8 T cells recovered was increased (**Figure 5.13**) it would seem on a per cell basis the ability to kill target cells was lower in the presence of IL-2 deficient CD4 T cells. Earlier studies showed that F5 IL-2 deficient CTL were unable to mediate cytolytic activity upon peptide immunisation, suggesting the induction

of cytotoxic function is IL-2 dependent, our data presented here is consistent with this publication (Kramer et al., 1994).

The data suggests that the improved killing of target cells upon transfer with WT CD4 T cells was IL-2 dependent however the expansion was not. The transfer of IL-2 deficient CD4 T cells showed there was no benefit from transfer of these cells because the level of target cell lysis by F5 memory CD8 T cells in the absence of CD4 T cells was equivalent. The proportion of CD8 T cells recovered from the recipient mice may not have been F5 CD8 T cells as discussed above, a possible explanation as to why there was no improvement in killing despite the increased cell numbers.

5.11: Discussion

This Chapter we addressed the role of Lck in the generation and maintenance of F5 memory CD8 T cells, and the ability of Lck to signal effector function. The data obtained from the studies conducted in **Chapters 3 and 4** showed that effector F5 CD8 T cells required the expression of Lck for optimal priming and the ability to mediate effector functions. Here we have shown the level of Lck expression and hence degree of optimal priming determines the magnitude of the response in terms of expansion and eliciting some effector functions of F5 memory CD8 T cells. We also demonstrate, in agreement with Tewari et al, that Lck is required for the activation and expansion of naïve F5 CD8 T cells, but was not required for the survival and hence maintenance of the memory F5 CD8 T cell compartment (Tewari et al., 2006).

We measured various cell surface expression markers including CD44, CD62L and CD43, which have been used to identify memory CD8 T cell populations (Galvan et al., 1998; Harrington et al., 2000; Kaech et al., 2002a; Sallusto et al., 1999). We observed some small differences in the expression of CD43 and CD62L, overall we can conclude that we were able to generate memory phenotype F5 CD8 T cells in the absence of Lck expression albeit at lower frequencies compared with F5 memory CD8 T cells which expressed Lck during priming (**Figure 5.3 and 5.8**). Other markers have also been identified as important indicators for those cells, which are potentially programmed to differentiate into memory cells. One such example is the IL-7R α (CD127). Studies have suggested that the expression of IL-7R α , in the primary phase of response determines the proportion of CD8 T cells, which will subsequently differentiate into memory CD8 T cells (Buentke et al., 2006; Kaech et al., 2003). We have not measured the expression of IL-7R α in our study and this would be an interesting marker to measure in order to assess the presence of memory cell precursors.

Previous studies have also shown that memory phenotype CD8 T cells selectively expressed high levels of CD122 (IL-2/IL-15R β), a receptor subunit shared by both IL-2 and IL-15 receptors (Zhang et al., 1998c). It has been shown that IL-15 causes selective proliferation of CD44 high CD8 T cells. In addition, studies from mice deficient in IL-15R α showed that expression of the IL-15R α was important for the survival of memory CD8 T cells (Lodolce et al., 1998) and is also important for the homeostatic proliferation of the memory CD8

T cell compartment (Becker et al., 2002). We have not measured the cell surface expression of either IL-7R α or IL-15R α/β and in further studies it would be interesting to see if the expression of these markers are different in F5 memory CD8 T cells which express low levels or no Lck, which could in turn influence the proportion of effector F5 CD8 T cells able to generate into memory cells.

Studies by Tewari et al have shown that Lck was not required for the secondary activation of memory CD8 T cells (Tewari et al., 2006). Upon secondary challenge with virus in the absence of Lck memory CD8 T cells proliferated to the same extent as Lck-sufficient memory CD8 T cells and showed similar levels of IFN γ and granzyme B production (Tewari et al., 2006). In contrast we have shown that F5 memory CD8 T cells do require the expression of Lck for eliciting effector functions such as killing target cells. However we have not strictly addressed whether Lck expression is required for secondary recall responses. In future experiments it would be interesting to see what the requirement for Lck is in the proliferation of F5 memory CD8 T cells and generation of effector function upon re-challenge with virus.

The concept of CD4 help in the generation of memory CD8 T cells has been around for some time now where it has been suggested that CD4 T cells are essential for the generation of memory CD8 T cells. Data shows that in the absence of CD4 T cells during the priming phase, CD8 T cells show poor proliferation (Bourgeois et al., 2002a; Shedlock and Shen, 2003; Sun and

Bevan, 2003) and the number of CD8 memory T cells was diminished when formed in the absence of CD4 T cells (Sun et al., 2004). The expansion of memory CD8 T cells upon antigen recall has been shown to be CD4 dependent (Marzo et al., 2004), however other models have shown in contrast that CD8 memory T cells are able to expand and elicit effector function in the absence of CD4 help (Shedlock and Shen, 2003; Sun and Bevan, 2003). Most of our experiments were conducted in lymphopenic recipient mice and thus in the absence of CD4 T cells at the time of priming. We therefore wanted to address the contribution of CD4 help in the generation of F5 memory CD8 T cells. We show that more F5 memory CD8 T cells were recovered after 5 weeks, with WT CD4 T cell co-transfer and there was also an improvement in cytolytic function (**Figure 5.13 & 5.15**). We hypothesised that this improvement may have been because the CD4 T cells were providing an additional source of IL-2 in order to improve the expansion.

In recent studies the expansion and survival of memory CD8 T cells have been shown to be helped by CD4 T cells through the secretion of IL-2 (Shi et al., 2006; Williams et al., 2006) and importantly IL-2 signalling during the primary response to acute infection was shown to help memory CD8 T cells to survive and accumulate following secondary exposure (Williams et al., 2006). To test whether IL-2 was helping F5 CD8 T cell memory generation and subsequent cytolytic function, we co transferred IL-2 deficient CD4 T cells with F5 CD8 T cells and showed that there were similar levels of expansion of F5 CD8 T cells in comparison to those which were transferred with WT CD4 T cells. This

suggested that the improvement in expansion was not because of IL-2. By contrast the degree of target cell killing was equivalent to those F5 CD8 T cells primed in the absence of CD4 help, suggesting killing was improved by IL-2. IL-2 deficient mice show severe lymphoproliferation and develop autoimmunity (Sadlack et al., 1993; Schorle et al., 1991), due to a breakdown in the regulation of T cells because IL-2 deficient mice lack T_{Reg} cells (Papiernik et al., 1998). Therefore one explanation for this expansion is that these cells are our donor IL-2 deficient. Expansion of CD8 T cells transferred with IL-2 deficient CD4 T cells, if proven to be F5 cells could be due to disrupted regulation. We have shown that F5 Lck^{ind} OFF memory CD8 T cells are able mediate some target cell lysis, therefore one would predict that more lysis would be observed in the presence of greater numbers of cells. An alternative and more likely reason for these results is that the CD8 T cells, which were recovered from the recipient mice, were not F5 memory CD8 T cells.

The increased expansion of F5 CD8 T cells in the presence of WT CD4 T cell help could be for two reasons. Firstly it could be because the CD4 T cells are providing an additional source of IL-2 helping the proliferation of activated F5 CD8 T cells as has been discussed. Or they could be providing help by cellular interactions. For example via CD40-CD40L interactions, CD4 T cells can activate DCs to upregulate co-stimulatory, surface MHC molecules and produce IL-12. Together these factors could enhance the stimulatory capacity of the DC to improve the presentation of antigen to F5 CD8 T cells resulting in improving priming and activation leading to an enhanced expansion of CD8 T cells

(Bourgeois et al., 2002a). We have shown that WT CD4 help improves cytolytic function, which appeared to be IL-2 dependent (**Figure 5.13**). Recent studies support the theory that CD4 help is being provided by a combination of both cell-cell interactions and also the production of IL-2 (Blachere et al., 2006). Although F5 Lck^{ind} OFF memory CD8 T cells showed no improvement in cytolytic function, supporting the theory that impaired priming and subsequent differentiation of F5 Lck^{ind} OFF CD8 T cells resulted in the defect of target cell killing, this defect could not be rescued with additional help from CD4 T cells.

In future studies it would be interesting to see to what extent co-transfer of CD4 T cells with F5 Lck^{ind} OFF CD8 T cells can compensate for the absence of Lck signalling. In Chapter 4 we demonstrated F5 Lck^{ind} OFF CD8 T cells did not show any proliferation in response to antigen until day 8 following priming (**Figure 4.2 & 3**). Does the presence of CD4 T cell help overcome the defect in priming of F5 Lck^{ind} OFF CD8 T cells leading to sufficient expansion and differentiation of F5 CD8 T cells? With CD4 help can F5 Lck^{ind} OFF CD8 T cells meet the threshold for activation and subsequently upregulate the early activation markers CD69 and CD25 leading to the differentiation into effector cells, which can produce effector proteins such as granzyme B. In addition it should be noted that the studies by Tewari et al were conducted in the presence of CD4 T cells. Does the presence of CD4 T cells, help F5 memory CD8 T cells upon re-challenge with flu virus?

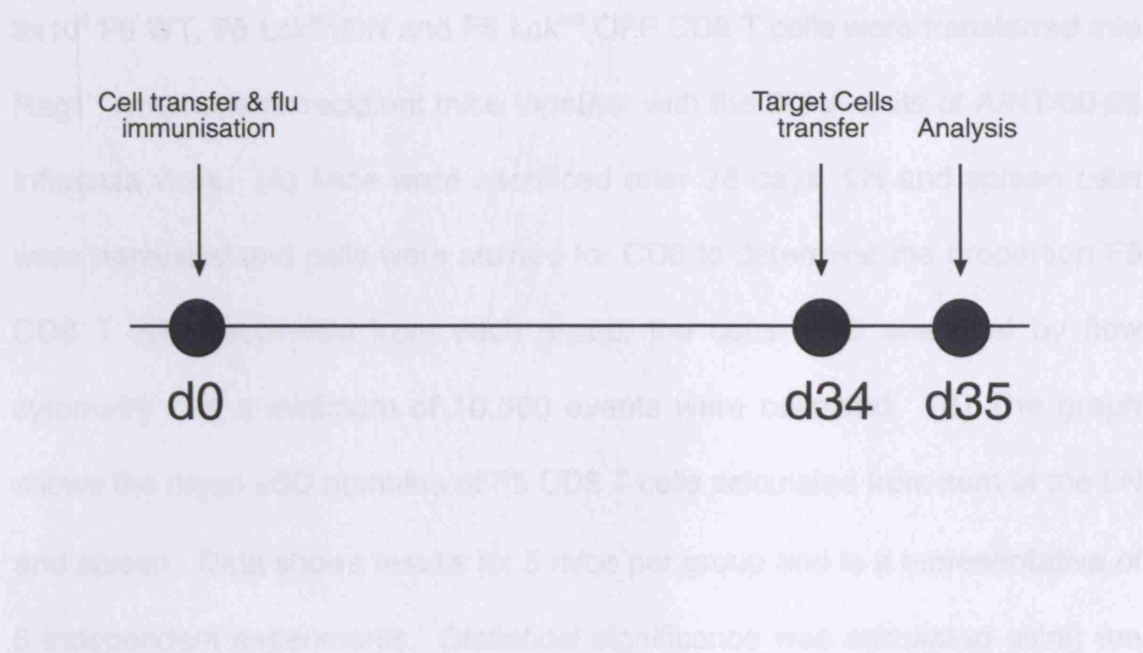
Figure 5.1: Timeline to illustrate the protocols used to investigate memory cell generation

(A) F5 WT, F5 Lck^{ind} ON and F5 Lck^{ind} OFF CD8 T cells were transferred into Rag1^{-/-} recipient mice together with 20HA units of influenza, A/NT-60-68 virus. Day 34 peptide pulsed target cells were transferred into the recipient mice. Day 35 the recipient mice were sacrificed, LN and spleen cells were harvested and the cells were analysed.

(B) F5 CD8 T cells were transferred with the influenza virus into the recipient mice. At day 7 and 28 post transfer, dox feed was removed from some groups of recipient mice (F5 Lck^{ind} OFF d7 and F5 Lck^{ind} OFF d28). At day 34 target cells were transferred into the recipient mice and at day 35 the mice were sacrificed and LN and spleen cells were harvested and cells were analysed.

Figure 8.2: Reduced numbers of memory CD8 T cells are recovered in the absence of Lck

A



B

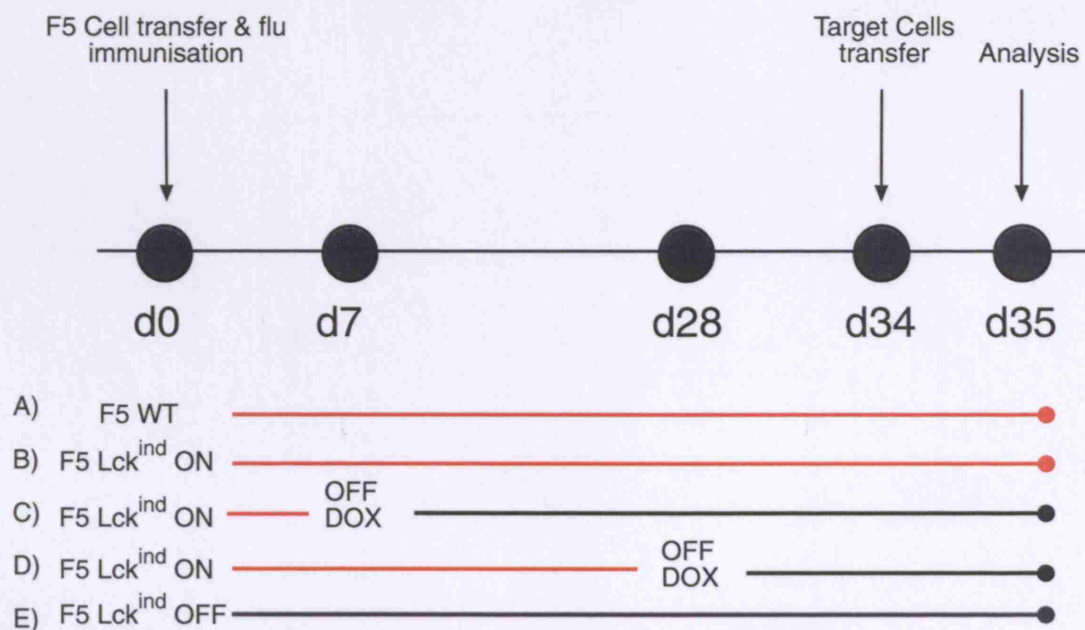
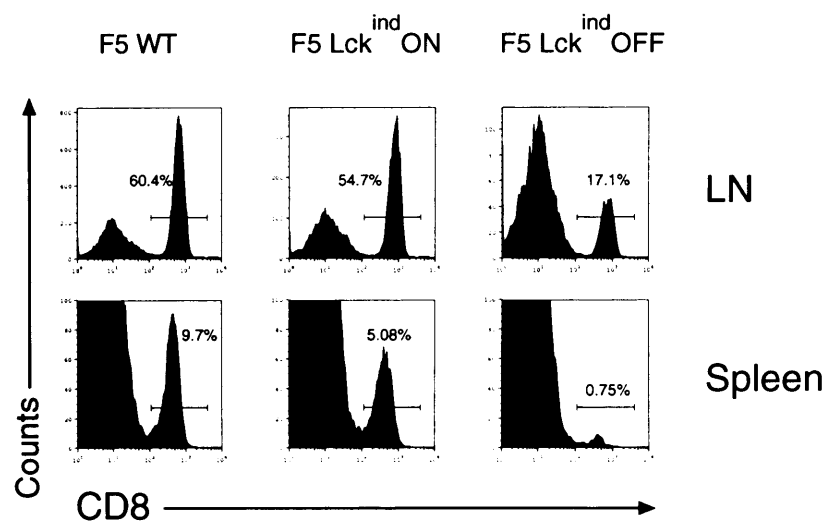


Figure 5.2: Reduced numbers of memory CD8 T cells are recovered in the absence of Lck

3×10^6 F5 WT, F5 Lck^{ind} ON and F5 Lck^{ind} OFF CD8 T cells were transferred into Rag1^{-/-} lymphopenic recipient mice together with the 20HA units of A/NT/60-68 influenza virus. (A) Mice were sacrificed after 35 days, LN and spleen cells were harvested and cells were stained for CD8 to determine the proportion F5 CD8 T cells recovered from each group, the cells were analysed by flow cytometry and a minimum of 10,000 events were collected. (B) The graph shows the mean \pm SD numbers of F5 CD8 T cells calculated from sum of the LN and spleen. Data shows results for 5 mice per group and is a representative of 5 independent experiments. Statistical significance was calculated using the Mann Whitney test ($P < 0.01 = **$)

A



B

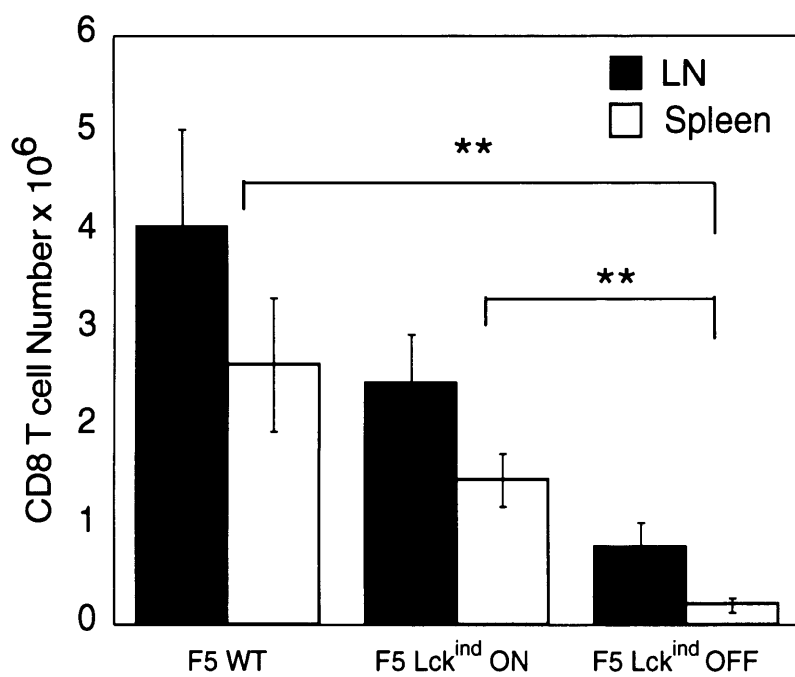


Figure 5.3: Memory Phenotype CD8 T cells are generated in the absence of Lck

(A) F5 WT, F5 Lck^{ind} ON and F5 Lck^{ind} OFF LN and spleens were stained with TCR and CD44 antibodies. LN cells were stained for (B) CD44 & CD62L and (C) CD44 & CD43. All cells were analysed by flow cytometry and a minimum of 10,000 events were collected. Data shows results for 5 mice per group and is representative of 3 independent experiments.

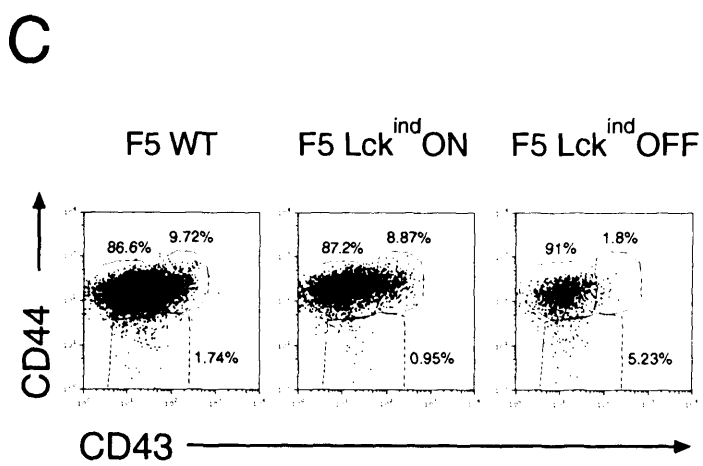
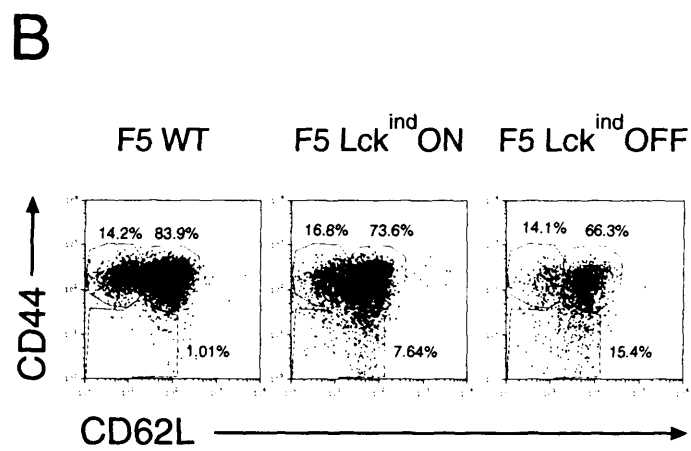
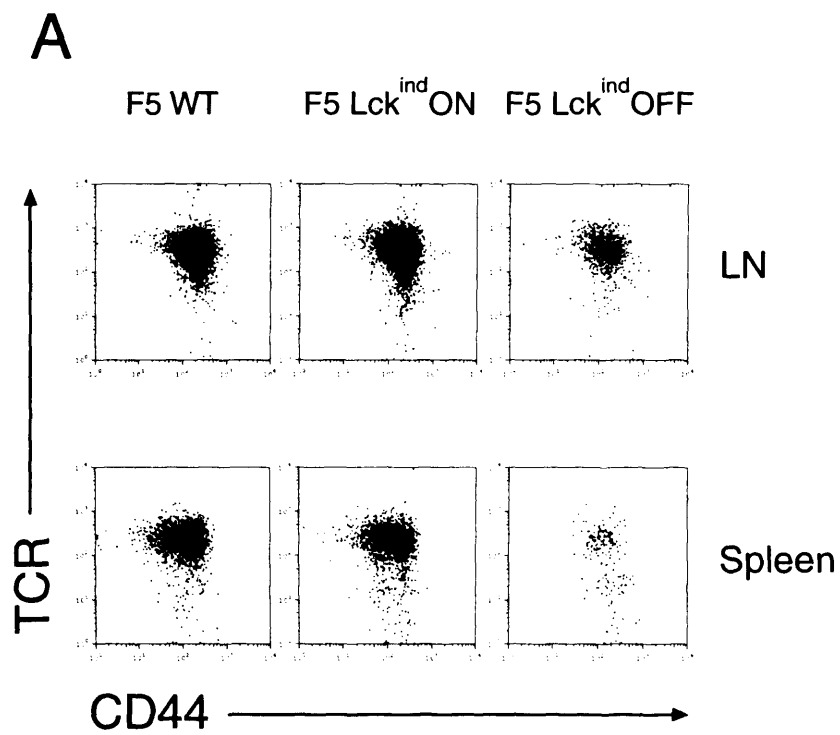
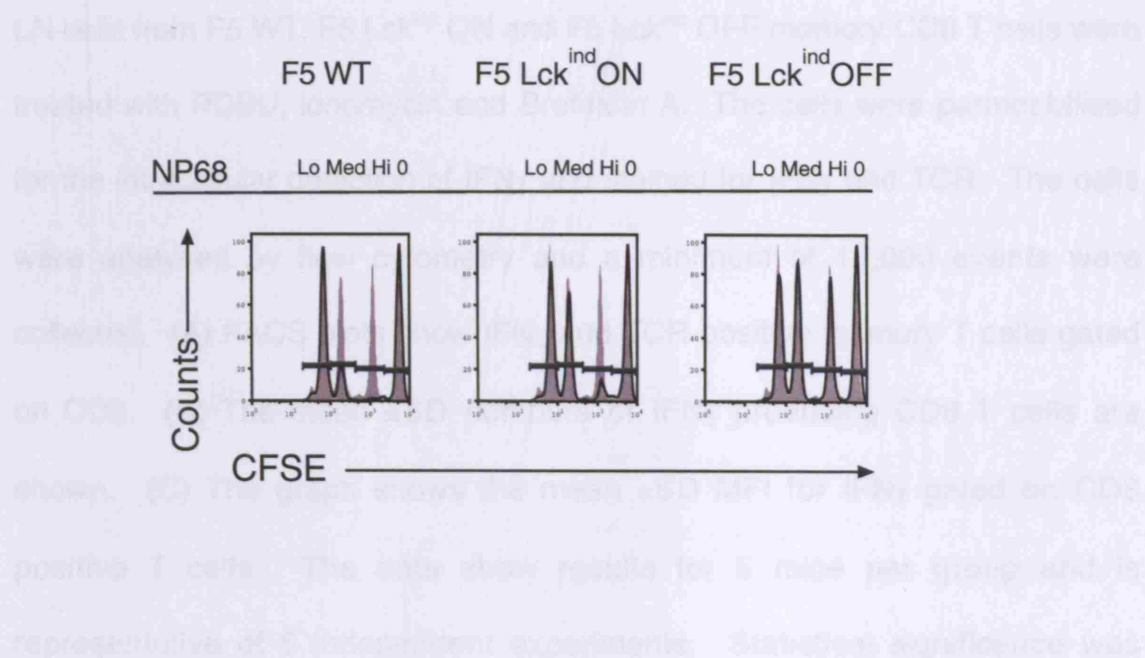


Figure 5.4: Memory CD8 T cells require Lck to kill target cells upon re-activation

3×10^6 F5 WT, F5 Lck^{ind} ON and F5 Lck^{ind} OFF CD8 T cells were transferred into Rag1^{-/-} recipient mice together with 20HA units of influenza A/NT/60-68 virus. At d34 LN derived target cells from C57BL/6 CD45.1 were harvested and pulsed with different concentrations of NP68 and CFSE and transferred into the recipient mice and into control naïve F5 Rag1^{-/-} mice. (A) FACS plots showing target cells recovered from experimental (black line) over target cells harvested from the F5 Rag1^{-/-} naïve control mice (shaded). Peak 0; (0 NP68 + 1.25 μ M CFSE), Peak Hi, (2.5 μ M NP68 + 0.25 μ M CFSE), Peak Med (2.5 $\times 10^{-3}$ μ M NP68 + 0.05 μ M CFSE), Peak Lo, (2.5 $\times 10^{-6}$ μ M NP68 + 0 CFSE). (B) The graph shows the calculated NP68 specific lysis of target cells as described in methods and materials section 2.9.3. The data shows results from 5 mice per group and is representative of 5 independent experiments. Statistical significance was calculated using the Mann Whitney test ($P < 0.01 = **$)

Figure 3.6: Reduced IFN γ production by memory CD8 T cells in the absence of Lck upon re-activation

A



B

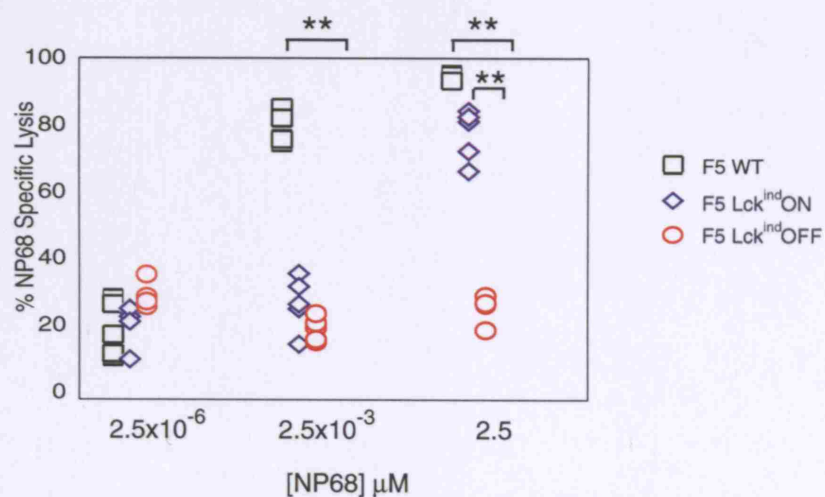
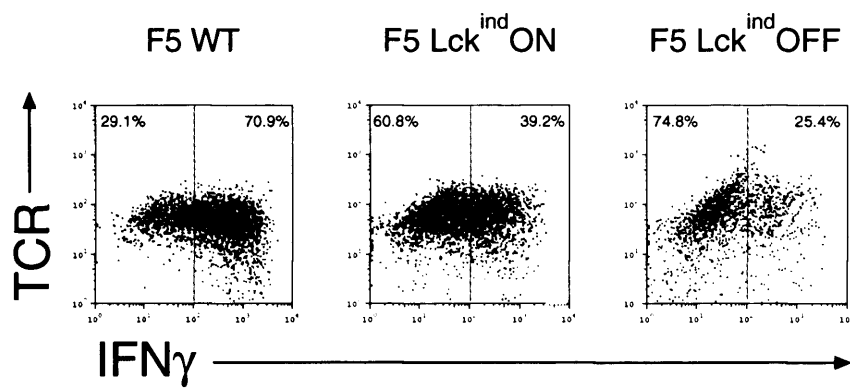


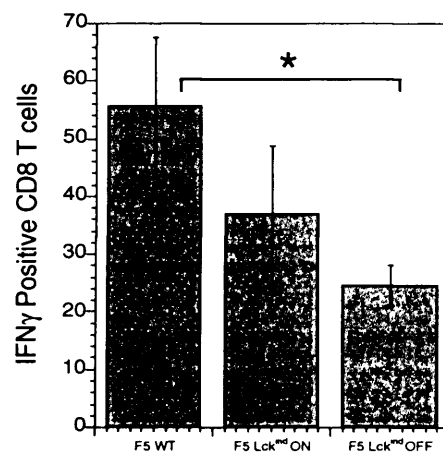
Figure 5.5: Reduced IFN γ production by memory CD8 T cells in the absence of Lck upon re-activation

LN cells from F5 WT, F5 Lck^{ind} ON and F5 Lck^{ind} OFF memory CD8 T cells were treated with PDBU, ionomycin and Brefeldin A. The cells were permeabilised for the intracellular detection of IFN γ and stained for IFN γ and TCR. The cells were analysed by flow cytometry and a minimum of 10,000 events were collected. (A) FACS plots show IFN γ and TCR positive memory T cells gated on CD8. (B) The mean \pm SD numbers of IFN γ producing CD8 T cells are shown. (C) The graph shows the mean \pm SD MFI for IFN γ gated on CD8 positive T cells. The data show results for 5 mice per group and is representative of 5 independent experiments. Statistical significance was calculated using the Mann Whitney test ($P < 0.05 = *$).

A



B



C

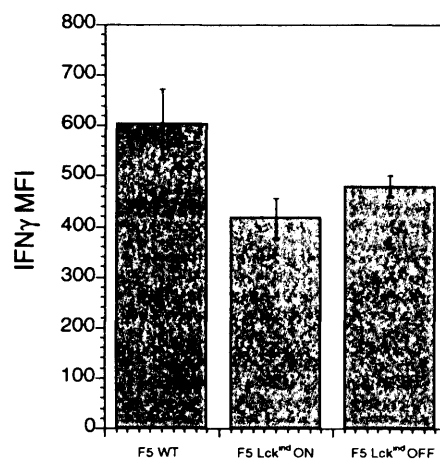
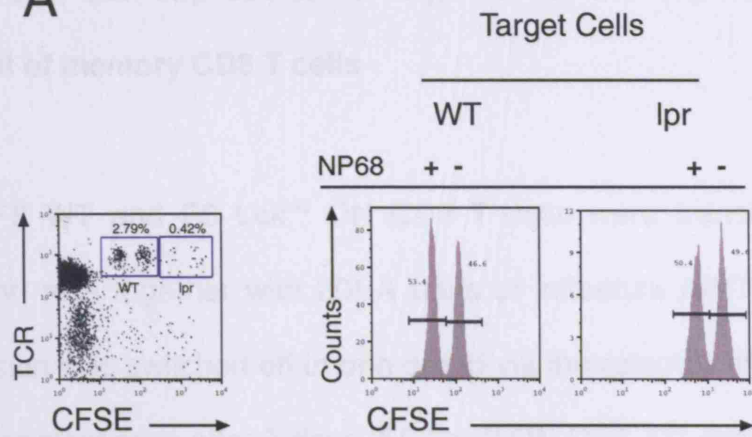


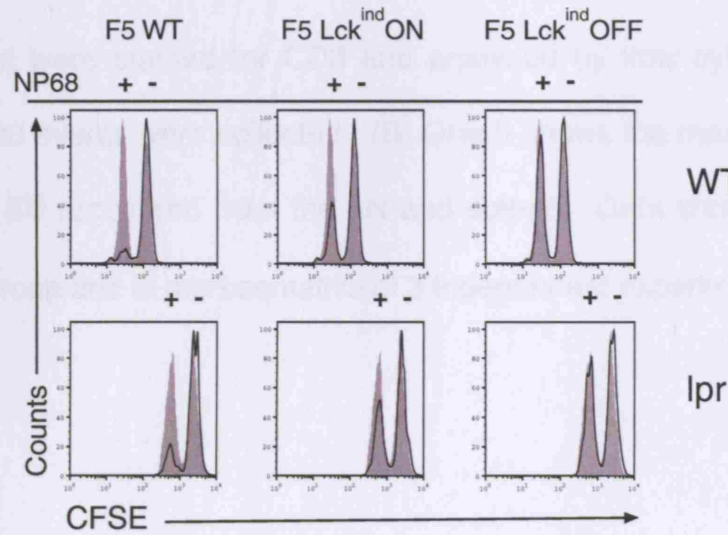
Figure 5.6: Fas/FasL pathway is not the major mechanism by which CTL mediate target cell lysis

F5 WT, F5 Lck^{ind} ON and F5 Lck^{ind} OFF were transferred into Rag1^{-/-} recipient mice together with 20HA units of influenza A/NT/60-68 virus. At d34 target cells were obtained from LNs of C57BL/6 and C57BL/6.MRL-Fas^{lpr} mice were transferred into the recipient and naïve F5 Rag1^{-/-} control mice. Target cells were divided into two groups one group was pulsed with 2.5μM NP68 and the other group was left unpulsed. Each population was labelled with different concentrations of CFSE. *lpr* target cells : peak + (CFSE 1.25μM) and peak – (CFSE 0.25μM). WT target cells: peak + (CFSE 0.05μM) and peak – (CFSE 0.01μM) and the four populations were injected in equal numbers. Recipient mice were sacrificed at d35 and LN cells were harvested. (A) FACS plots show the target cell populations recovered from the naïve F5 Rag1^{-/-} control mice. (B) FACS plots show the targets cells recovered from the recipient mice from each group (black line) superimposed onto the target cell profiles from the control mice (filled grey histograms) to show target cell lysis relative to the control. (C) The graph shows the calculated NP68 specific lysis of WT and *lpr* target cells. (D) The graph shows the percentage of FasL positive CD8 T cells from a related experiment. The data shows results from 5 mice per group and is representative of 2 independent experiments.

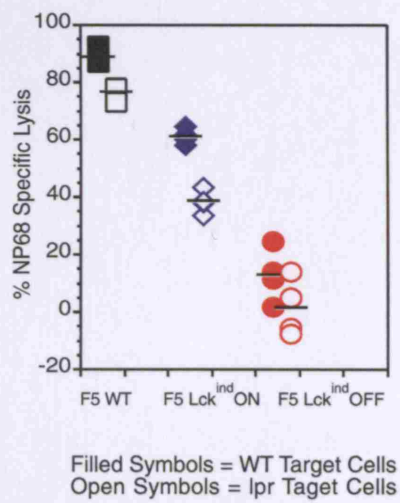
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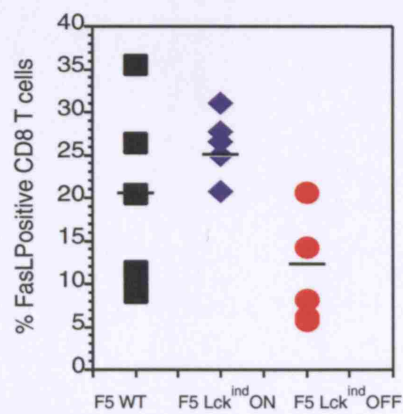
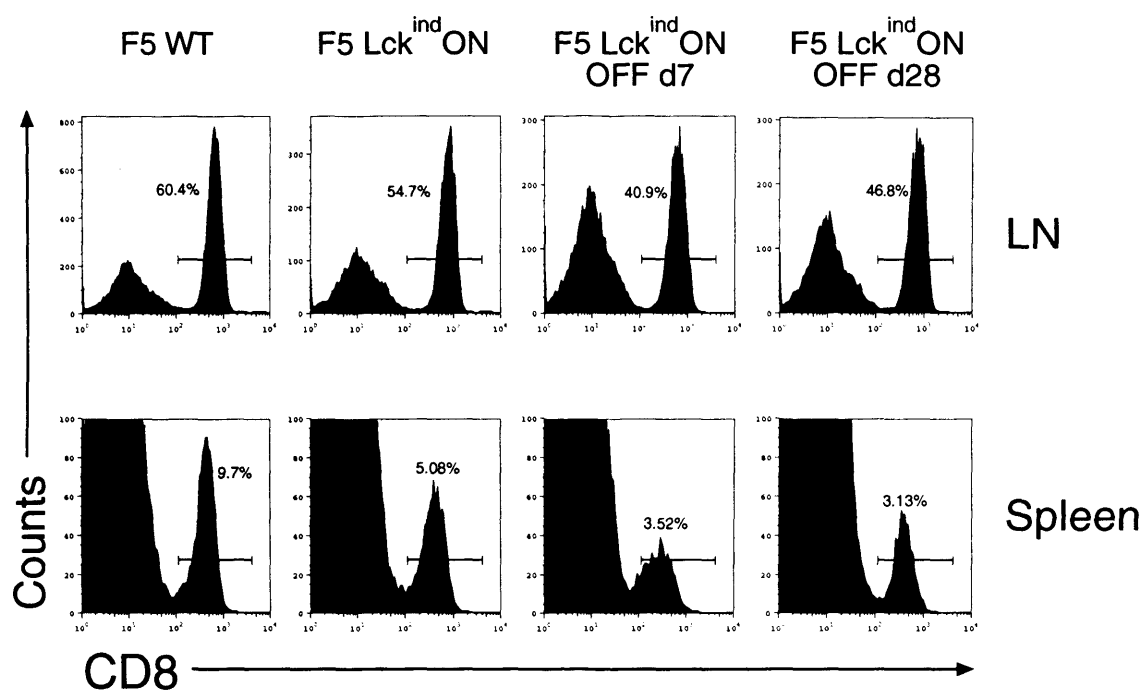


Figure 5.7: Lck expression is required for the expansion but not the survival of memory CD8 T cells

3×10^6 F5 WT and F5 Lck^{ind} ON CD8 T cells were transferred into Rag1^{-/-} recipient mice together with 20HA units of influenza A/NT/60-68 virus. Lck expression was switched off in one group via the removal of dox from the feed of the recipient mice after 7 days (F5 Lck^{ind} ON OFF d7) and in a second group after 28 days (F5 Lck^{ind} ON OFF d28). (A) LN and Spleen cells were harvested after 35 days and were stained for CD8 and analysed by flow cytometry, a minimum of 10,000 events were collected. (B) Graph shows the mean number of CD8 T cells \pm SD recovered from the LN and spleen. Data shows results from 5 mice per group and is representative of 3 independent experiments.

A



B

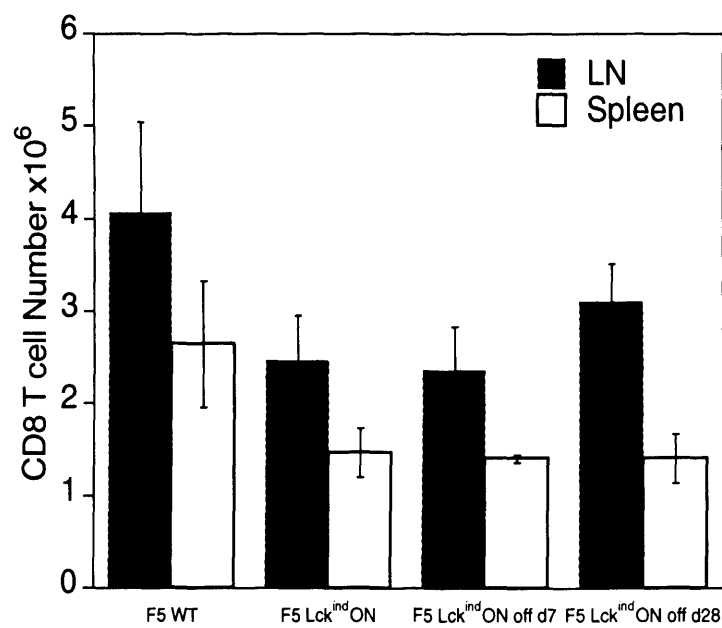
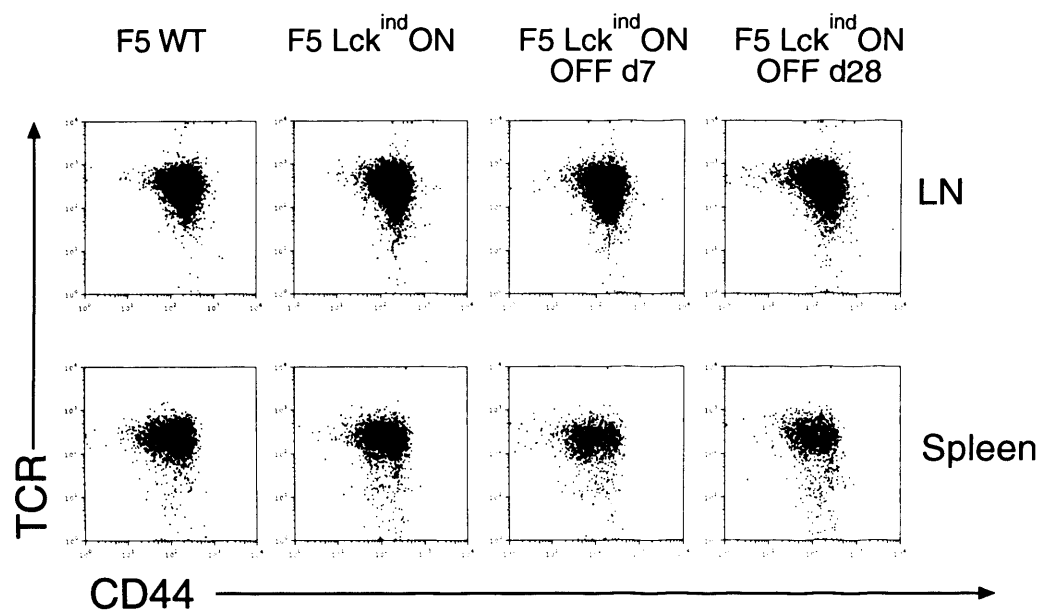


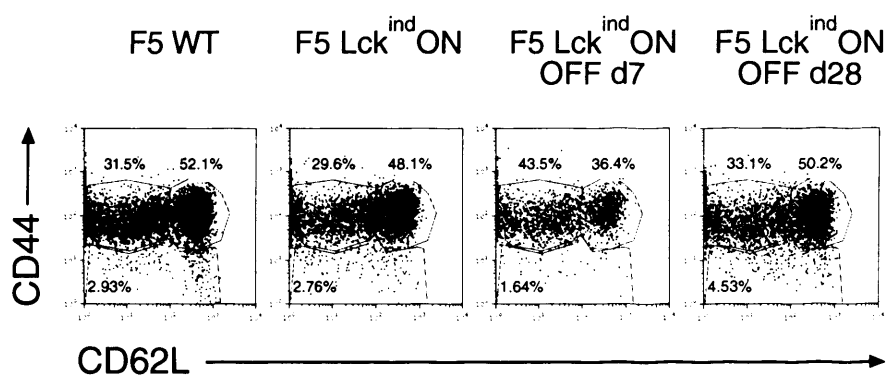
Figure 5.8: Phenotype of memory CD8 T cells is not altered by controlling the expression of Lck

(A) F5 WT, F5 Lck^{ind} ON, F5 Lck^{ind} ON OFF d7 and F5 Lck^{ind} ON OFF d28 LN and spleens were stained for TCR and CD44. LN cells were stained for CD44 and CD62L (B) and CD43 (C). All cells were analysed by flow cytometry and a minimum of 10,000 events were collected. Data shows one representative FACS plot from each group. There were 5 mice per group and data is representative of 3 independent experiments.

A



B



C

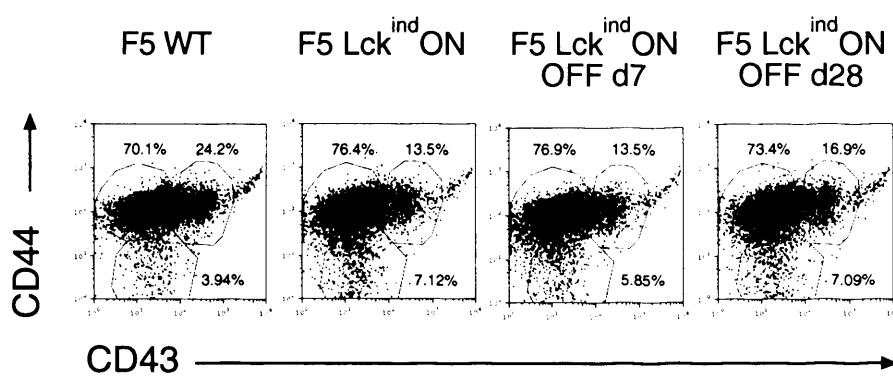


Figure 5.9: Signals through Lck are required for the cytolytic function of re-activated memory CD8 T cells

3 x10⁶ F5 WT and 3 groups of F5 Lck^{ind} ON CD8 T cells were transferred into Rag1^{-/-} recipient mice together with 20HA units of influenza A/NT-60-68 virus. Lck expression was switched off by the removal dox from the feed of the recipient mice in one group after 7 days (F5Lck^{ind} ON OFF d7) and in the other group after 28 days (F5 Lck^{ind} OFF d28). (A) LN derived targets cells from C57BL/6 CD45.1 mice were transferred into the recipient mice and naïve F5 Rag1^{-/-} control mice at d34 and at d35 the mice were sacrificed for analysis. (B) FACS plots show the target cells recovered from the recipient mice superimposed onto target cells recovered from the naïve F5 Rag1^{-/-} control mice to show target cell lysis relative to the control. (C) The graph shows the calculated NP68 specific lysis of the target cells. The data shows results from 5 mice per group and is representative of 3 independent experiments.

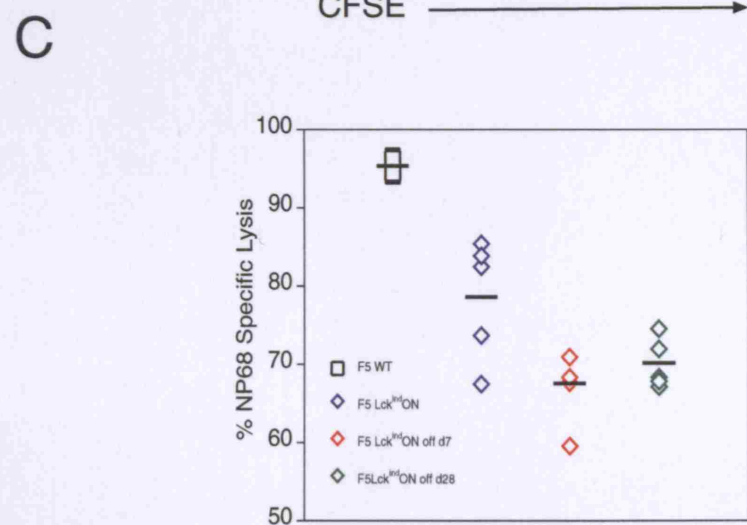
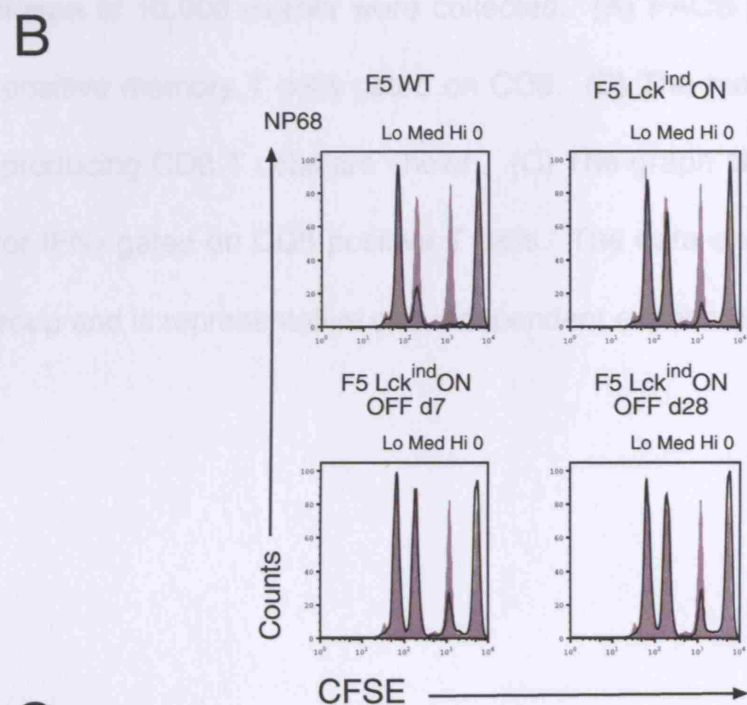
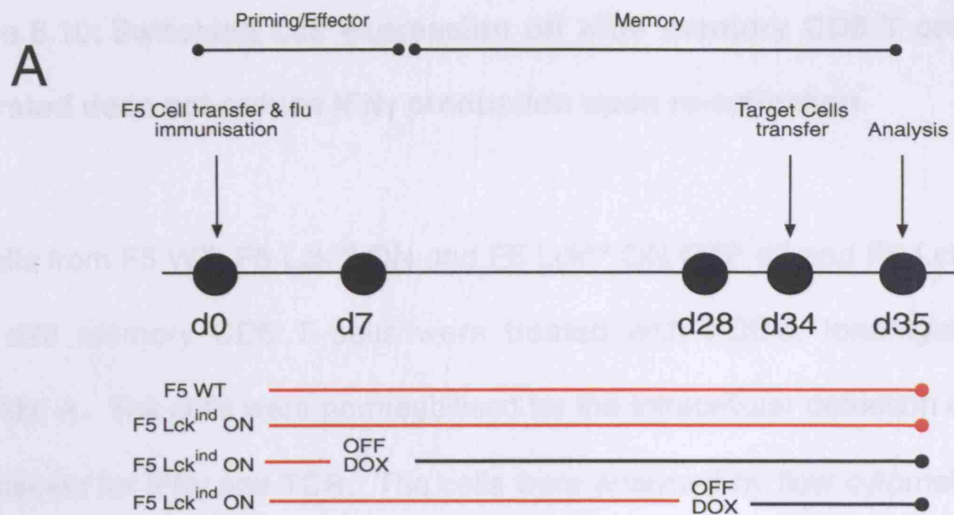
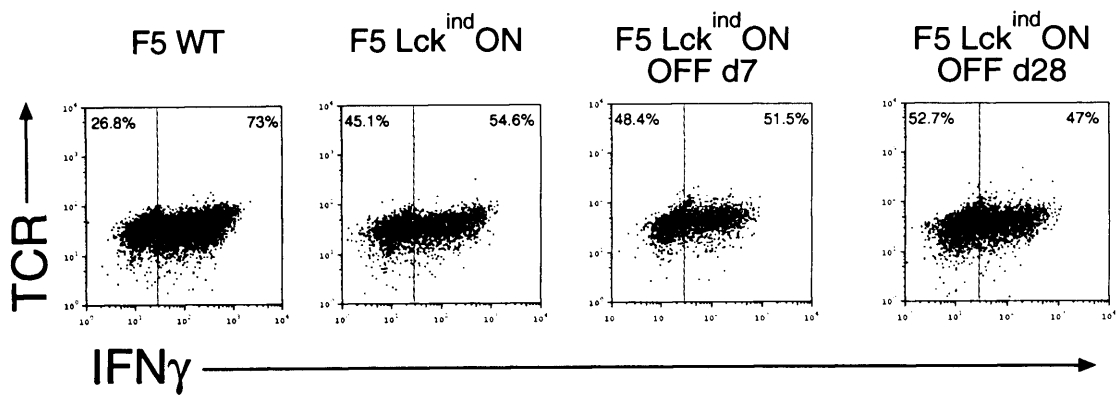


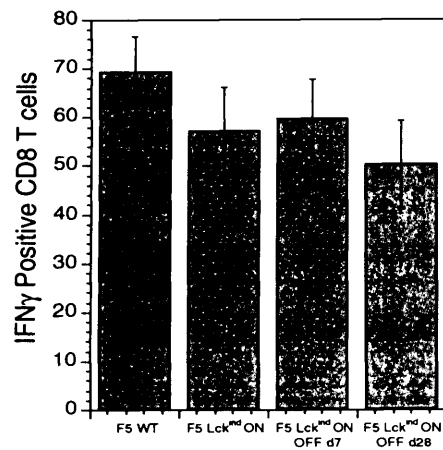
Figure 5.10: Switching Lck expression off after memory CD8 T cells are generated does not reduce IFN γ production upon re-activation

LN cells from F5 WT, F5 Lck^{ind} ON and F5 Lck^{ind} ON OFF d7 and F5 Lck^{ind} ON OFF d28 memory CD8 T cells were treated with PDBU, ionomycin and Brefeldin A. The cells were permeabilised for the intracellular detection of IFN γ and stained for IFN γ and TCR. The cells were analysed by flow cytometry and a minimum of 10,000 events were collected. (A) FACS plots show IFN γ and TCR positive memory T cells gated on CD8. (B) The mean \pm SD numbers of IFN γ producing CD8 T cells are shown. (C) The graph shows the mean \pm SD MFI for IFN γ gated on CD8 positive T cells. The data show results for 5 mice per group and is representative of 3 independent experiments.

A



B



C

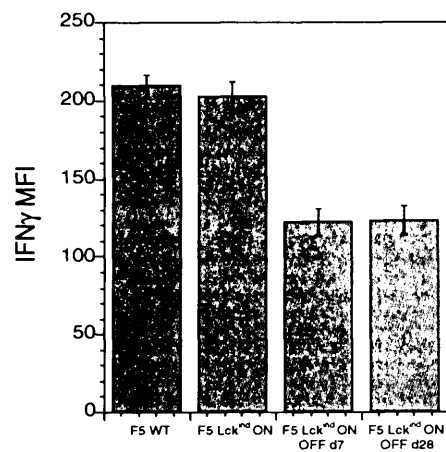
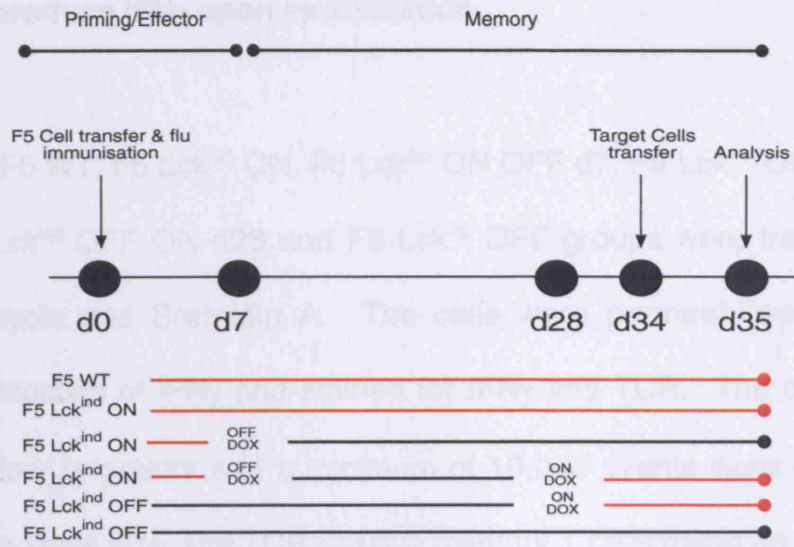


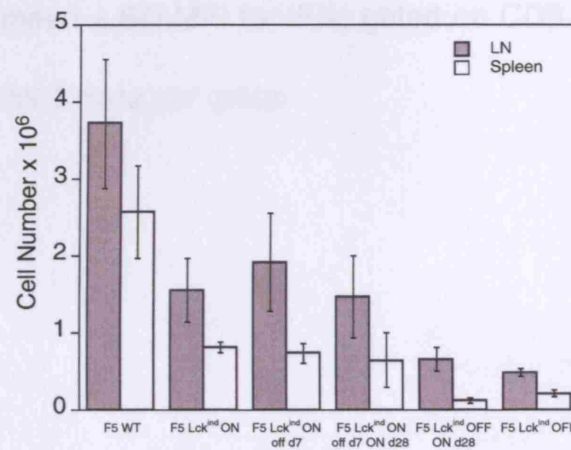
Figure 5.11: Switching Lck expression on in F5 Lck^{ind} OFF memory CD8 T cells restores cytolytic function to equivalent F5 Lck^{ind} ON levels

(A) A scheme for the experiment shows: 3×10^6 F5 WT, 3 groups of F5 Lck^{ind} ON and 2 groups of F5 Lck^{ind} OFF CD8 T cells were transferred into Rag1^{-/-} recipient mice together with 20HA units of influenza A/NT-60-68 virus. Lck expression was switched off by the removal of dox from the feed of the recipient mice in 2 groups after 7 days (F5Lck^{ind} ON OFF d7) and subsequently switched back on in one group at day 28 (F5 Lck^{ind} OFF d7 ON d28). Lck expression was also switched back on in one F5 Lck^{ind} OFF group by the re-introduction of dox in the feed of the recipient mice at day 28 (F5 Lck^{ind} OFF ON d28). Peptide pulsed target cells from C57BL/6 CD45.1 were transferred into the recipient mice and naïve F5 Rag1^{-/-} control mice at d34 and at d35 the mice were sacrificed for analysis. (B) Graph shows cell recoveries of F5 memory CD8 T cells from the recipient mice. (C) The graph shows the calculated NP68 specific lysis of the target cells. The data shows results from 5 mice per group. Statistical significance was calculated using the Mann Whitney test ($P < 0.05 = *$).

A



B



C

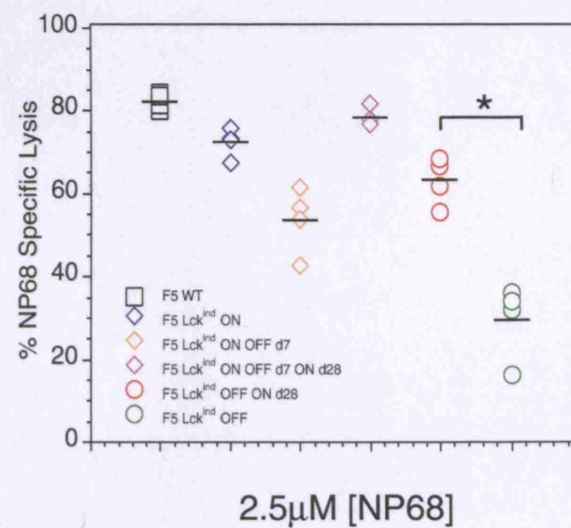
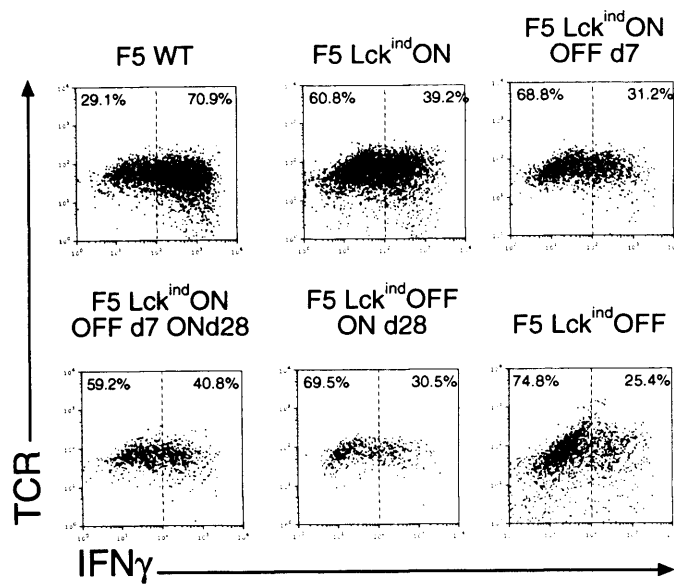


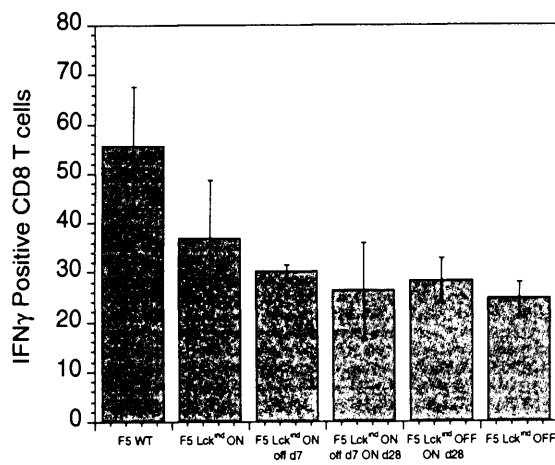
Figure 5.12: Lck expression does not alter the number of CD8 memory T cells able to produce IFN γ upon re-activation

LN cells from F5 WT, F5 Lck^{ind} ON, F5 Lck^{ind} ON OFF d7, F5 Lck^{ind} ON OFF d7 ON d28, F5 Lck^{ind} OFF ON d28 and F5 Lck^{ind} OFF groups were treated with PDBU, ionomycin and Brefeldin A. The cells were permeabilised for the intracellular detection of IFN γ and stained for IFN γ and TCR. The cells were analysed by flow cytometry and a minimum of 10,000 events were collected. (A) FACS plots show IFN γ and TCR positive memory T cells gated on CD8. (B) The mean \pm SD numbers of IFN γ producing CD8 T cells are shown. (C) The graph shows the mean \pm SD MFI for IFN γ gated on CD8 positive T cells. The data show results for 5 mice per group.

A



B



C

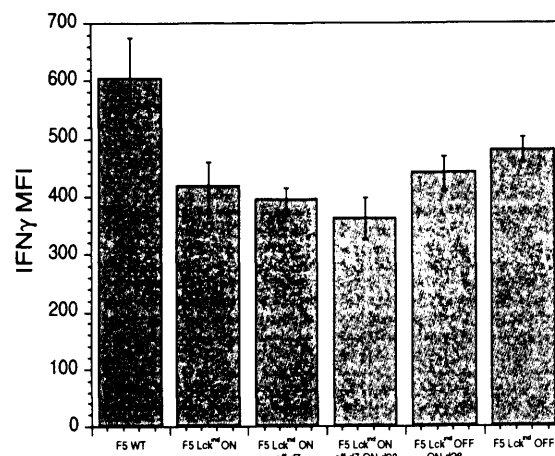
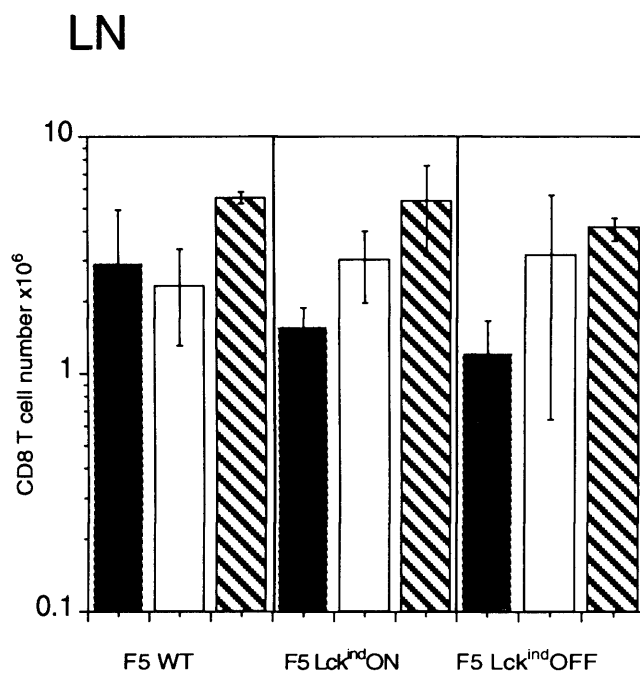


Figure 5.13: CD4 T cells enhance the expansion of activated CD8 T cells

3×10^6 F5 WT, F5 Lck^{ind} ON and F5 Lck^{ind} OFF were transferred into Rag1^{-/-} recipient mice together with the 20HA units of influenza A/NT/60-68 virus. Each group was co-transferred with 3×10^6 WT CD4, IL-2 deficient CD4 or no CD4 T cells. 35 days after cell transfer mice were sacrificed and LN (A) and Spleen (B) cells were harvested. The graphs show the mean number of CD8 T cells \pm SD recovered from the LN (A) and spleen (B). The data show results from 4 mice per group and is representative of 2 independent experiments. Statistical significance was calculated using the Mann Whitney test ($P < 0.05 = *$).

A



B

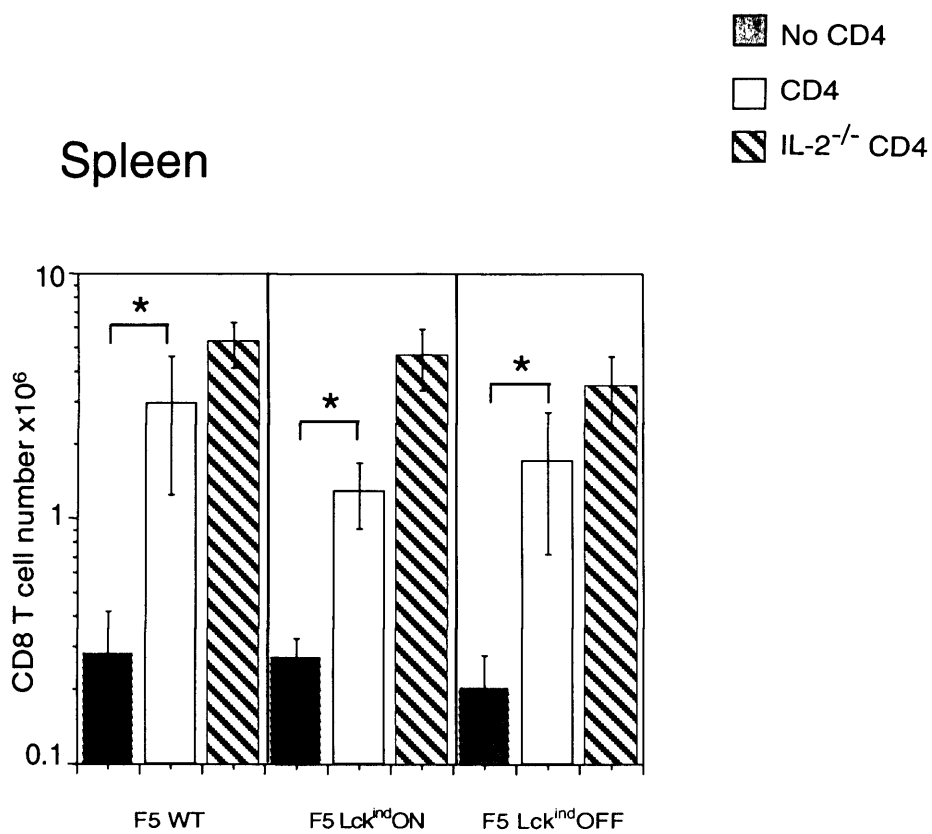


Figure 5.14: Phenotype of memory CD8 T cells generated in the presence of IL-2 deficient CD4 T cells is altered

LN cells from F5 WT, F5 Lck^{ind} ON and F5 Lck^{ind} OFF memory CD8 T cells generated in the presence of WT CD4, IL-2 deficient CD4 T or no CD4 T cell T cells were stained for TCR, CD44 (A) and CD43 (B) and were analysed by flow cytometry. A minimum of 10,000 events were collected, the data shows results from 4 mice per group and is representative of 2 independent experiments.

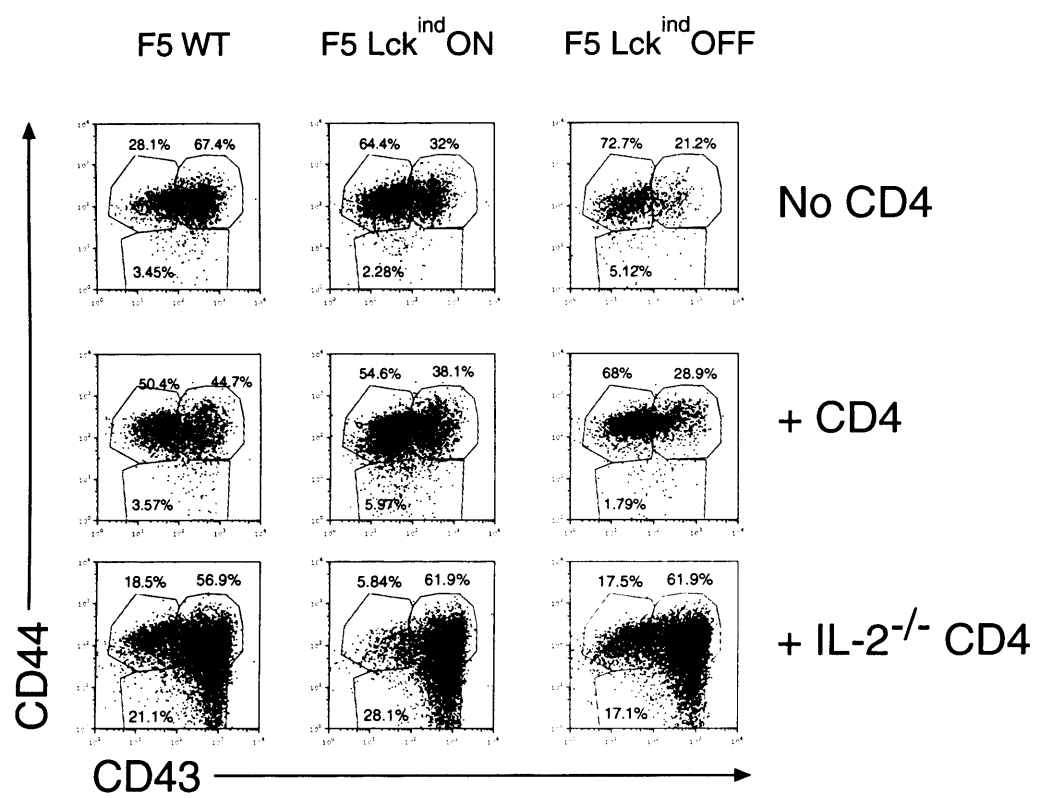
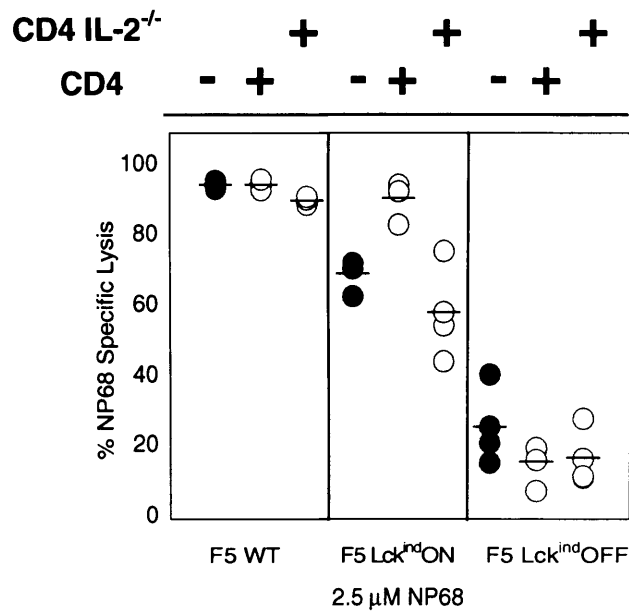


Figure 5.15: CD4 T cells improves memory CD8 T cell cytolytic function upon re-activation

3×10^6 F5 WT, F5 Lck^{ind} ON and F5 Lck^{ind} OFF were transferred into Rag1^{-/-} recipient mice together with the 20HA units of influenza A/NT/60-68 virus. Each group was co-transferred with 3×10^6 WT CD4, IL-2 CD4 or no CD4 T cells. LN derived target cells from CD57Bl/6 CD45.1 mice were transferred into recipient mice as previously described. The graph shows NP68 specific target cell lysis of F5 re-activated memory CD8 T cells at high (A) and intermediate (B) dose of peptide pulsed target cells. The data shows results for 4 mice per group and is representative of 2 independent experiments.

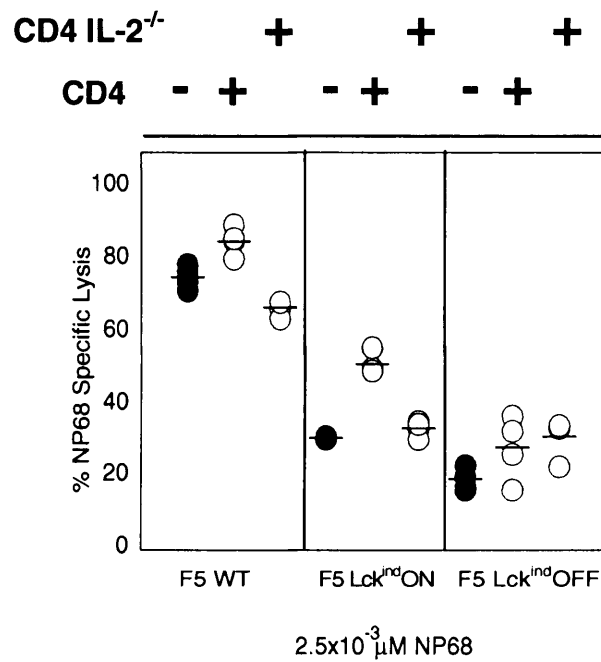
A

High peptide concentration on target cells



B

Medium peptide concentration on target cells



Chapter 6: Final Discussion

The overall aim of this study was to elucidate the role of Lck in peripheral CD8 T cells responses. We have shown with experiments conducted both *in vitro* and *in vivo* that Lck is required for the triggering of naïve F5 CD8 T cells, and their subsequent generation into functional effector and memory CD8 T cells. Upon stimulation of naïve F5 CD8 T cells *in vitro* with peptide, we showed the partial and complete absence of Lck raises the threshold for triggering the cells into division, resulting in impaired upregulation of early activation markers CD69 and CD25 (**Figure 3.4**) and production of cytokines IL-2, TNF α and IFN γ (**Figure 3.5 & 3.6**). In addition in the complete absence of Lck Granzyme B production was reduced and cytolytic function was severely impaired (**Figure 3.8**).

In vivo analysis confirmed that the activation and expansion of F5 CD8 T cells was Lck dependent (**Figure 4.2, 4.3 & 4.4**) and further showed that Lck was required to varying degrees to mediate effector function depending on the parameters measured. In the complete absence of Lck F5 CTL were severely impaired in their ability to kill target cells, but there was a less stringent requirement for Lck for the production of IFN γ (**Chapter 4 & 5**). In addition we showed that the re-expression of Lck in F5 memory CD8 T cells generated in the absence of Lck restored lytic function (**Figure 5.10**). Together the data showed that Lck is required during multiple stages of memory CD8 T cell development, in the priming, expansion and differentiation, but is not required

for their survival. Importantly our data has clearly demonstrated that Lck substantially influences the cytolytic function of both effector and memory F5 CD8 T cells. Finally we have shown that different thresholds are set to achieve particular effector functions and Lck influences which thresholds are crossed.

6.1 Lck is required for F5 CD8 T cell survival

An incidental finding in our study was the decline in naïve T cell numbers following cessation of Lck expression in F5 Lck^{ind} OFF mice. Peripheral T cell numbers dropped 3 fold compared to F5 Lck^{ind} ON mice over 7 days. This observation pointed towards a requirement for Lck in naïve T cell survival. However studies with polyclonal mice showed that naïve T cell survival did not require Lck providing Fyn was expressed (Seddon and Zamoyska, 2002a). In addition to TCR mediated signals, IL-7 has also been shown to be important in naïve T cell survival (Schluns et al., 2000; Seddon and Zamoyska, 2002a). In polyclonal mice IL-7R α expression was not reduced in the absence of Lck or Fyn it was infact upregulated on CD4 T cells (Seddon et al., 2003). Therefore signals through both the TCR and IL-7R α were shown to be required for naïve T cell survival and in the absence of TCR signalling survival may be more dependent on IL-7R signalling. The expression of IL-7R α is however considerably reduced in F5 mice, for reasons we do not understand (Seddon and Zamoyska, 2002a). Therefore it is possible that the combination of the low level of IL-7R α expression and the absence of Lck in F5 Lck^{ind} OFF mice was affecting naïve T cell survival. The pro-survival factor Bcl-2 is expressed in resting naïve T cells (Broome et al., 1995), and in polyclonal mice the

expression of Bcl-2 in the partial and complete absence of Lck in naïve T cells is also normal, compared to WT mice (Seddon and Zamoyska, 2002a). However we have not measured the level of Bcl-2 expression in F5 mice. It is possible that the expression levels may also be reduced similarly to the IL-7R α expression levels. Bcl-2 expression has been shown to be impaired in the absence of IL-7 signalling upon activation of naïve T cells (Schluns et al., 2000). If IL-7R α expression is reduced, together with the reduced expression of Bcl-2 in the absence of Lck, this may explain why naïve F5 CD8 T cell survival is compromised. However it is interesting that, activated F5 Lck^{ind} OFF CD8 T cells can develop into memory cells and survive long term in the absence of Lck (**Figure 5.7**). Bcl-2 family members are important for maintaining T cell homeostasis, Bcl-2 itself promotes cell survival and other members such as Bim promote cell death (reviewed in (Marrack and Kappler, 2004)). In resting naïve T cells Bcl-2 and Bcl_{xL} have been shown to associate with Bim (Zhu et al., 2004). It has been suggested that reduction in Bcl-2 levels may lead to small increases in free Bim. Bim in turn may function to activate the pro apoptotic factors Bax and Bak, thus leading to cell death. Upon activation, T cells upregulate Bcl-2 expression prior to cell division, and upon division the cells progressively down regulate Bcl-2 expression (Gett et al., 2003). However to compensate they upregulate Bcl_{xL} expression, which has a redundant role with Bcl-2, and is also a survival factor (Boise et al., 1995; Gett et al., 2003). We hypothesise that in naïve F5 Lck^{ind} OFF T cells, Bcl-2 expression may be low, as is IL-7R α expression resulting in cell numbers declining over time, due to an increase in free Bim promoting cell death. However upon activation, the

stimulus may be sufficient to upregulate Bcl-2 expression in concert with Bclx_L to help the survival of the memory cells which have been generated. It would be interesting to measure the changes in the expression of pro- and anti-apoptotic factors in the course of the activation of F5 Lck^{ind} OFF CD8 T cells, to understand the difference between naïve cells, which appear to be dying upon removal of Lck, and memory cells which can survive long term.

One important difference between naïve and memory CD8 T cells is the latter is influenced by IL-15 and to a lesser extent IL-7 (Kennedy et al., 2000; Lodolce et al., 1998; Schluns et al., 2000). IL-15 binds to its high affinity receptor, IL-15R α (CD122) (Dubois et al., 2002). Therefore in addition to measuring the expression levels of different survival factors mentioned, it will also be useful measure the expression of CD122 in F5 memory CD8 T cells.

6.2 Lck is required for the cytolytic activity of F5 CD8 T cells

Our work has uncovered a strict requirement for Lck to elicit cytolytic activity of both effector and memory F5 CD8 T cells, although some basal killing function was observed in the absence of Lck. *In vivo* imaging studies have shown that an immunological synapse is formed between the CTL and target, and has a distinct structure (Stinchcombe et al., 2001). Signalling molecules cluster in a cSMAC and are surrounded by a pSMAC containing adhesion molecules such as LFA1. Upon contact with a target cell, CTL rapidly re-organise their membrane proteins to form an immunological synapse, enclosing a patch of signalling proteins containing TCR, Lck and PKC θ (Stinchcombe et al., 2001).

The CTL immunological synapse contains a second patch found in the adhesion ring, called the secretory domain, which is the site at which the CTL exposes the cytotoxic proteins contained within the secretory lysosome (SL). The data shows that the synapse structure is important in directing the secretion of the cytotoxic proteins. These studies also demonstrated that the areas of membrane contact and organisation of signalling proteins are maintained during secretion. Early studies also showed that TCR signalling in CTL not only stimulates the secretion of the cytotoxic proteins but also results in the synthesis of new lytic proteins, which can refill the granules (Isaaz et al., 1995).

It would be interesting to see if there is a disruption in the formation of the immunological synapse and subsequently a re-distribution of the signalling components, in the absence of Lck. Also we could try to understand if other molecules important in signal transduction can re-distribute, in order to compensate for the absence of Lck and if they do, then to what extent. Another interesting observation in our study showed F5 Lck^{ind} OFF memory CD8 T cells upon re-expression of Lck displayed better cytolytic function on a per cell basis than those memory cells in which Lck expression was maintained throughout their generation. It is possible that the signalling molecules rearrange themselves to compensate for the absence of Lck, and upon re-expression of Lck are more sensitive to antigen recognition. This could lead to a reduced threshold for activating cytolytic function in these F5 memory CD8 T cells. One way to address these points would be to conduct imaging studies.

6.3 Thresholds for activating different effector functions

In our studies we also concluded that different thresholds were set for activating different effector functions in F5 memory CD8 T cells. It seemed that the activation threshold to produce a small amount of IFN γ was lower than that required for cells to mediate cytolytic function. However higher levels of Lck expression was required to support production of WT levels of IFN γ . A correlation between the number of cell divisions and cytolytic activity has previously been reported, furthermore the acquisition of cytolytic function was shown to be dependent on cell division (Auphan-Anezin et al., 2003; Opferman et al., 1999). In agreement with these published studies that in the absence of Lck, F5 CD8 T cells, underwent reduced proliferation compared with Lck sufficient F5 CD8 T cells and showed impaired cytolytic function. In addition a link between CD8 T cell proliferation and cytokine production was also demonstrated where Auphan-Anezin et al showed that the activation threshold for the commitment to produce IFN γ and express CD44 was lower than that required for cell division and induction of cytolytic function (Auphan-Anezin et al., 2003). We suggest in agreement with Auphan-Anezin et al that the priming of CD8 T cells may be induced by suboptimal stimulation, which can lead to an incomplete differentiation program of CD8 T cells (**Chapter 3**). This would support the conclusion that different signalling thresholds can drive low level of IFN γ production compared with cell cycle entry and acquisition of cytolytic activity. However in the situation of suboptimal stimulation of T cells, a sustained or a secondary stimulus could replace a stronger and shorter signal. If so this could have important implications particularly in developing vaccines

against weak immunogenic antigens. Tewari et al showed that secondary activation of memory CD8 T cells with LCMV did not require Lck expression (Tewari et al., 2006). However since we have found that IFN γ production albeit to a lesser extent, and cytolytic activity does require the expression of Lck in F5 memory CD8 T cells. It would be interesting to see if re-challenge of F5 memory CD8 T cells with flu in the absence of Lck, results in cell proliferation and improved acquisition of effector function.

6.4 Clinical Application

Understanding the precise signalling mechanisms involved in the activation of naïve T cells and the generation of effector and memory T cells is particularly important in trying to understand the reasons and mechanisms by which the immune system can be challenged upon infection or disease.

A recent study has shown that following activation, CTLs can enter a state of activation-induced non-responsiveness that is characterised by the inability of T cells to respond to a secondary stimulus (Uhlir et al., 2005). They showed that TCR triggering resulted in the rapid downregulation of Lck. Uhlir et al also went on to show that pharmacological blocking of Lck degradation and the transfection of anergic CTLs with an Lck expression vector, increased the responsiveness of CTLs to antigen re-challenge. In addition, the administration of exogenous IL-15 and IFN α also restored both Lck expression and re-activation of CTLs. IL-15 has been shown to augment CTL responses to HIV antigens both in experimental vaccination models and in peripheral blood

mononuclear cells of AIDS patients, whereby the functional incapacity of HIV specific CTLs represents a critical factor in the pathogenesis of the disease (Chitnis et al., 2003; Oh et al., 2003; Seder et al., 1995). The upregulation of Lck in non-responsive CTLs may help to enhance their effector function. This can potentially be done by using pharmacological agents and cytokines in developing new therapies aimed at the immunological control of infections and disease.

Chapter 7: References

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